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13. ABSTRACT *(Maximum 200 Words)*

The failure to eradicate advanced prostate cancer that is resistant to conventional therapies has led to the exploration of immunotherapy. TRAIL is being considered as a novel therapeutic agent due to its selective cytotoxicity to cancer cells. However, prostate cancer cell lines are resistant to TRAIL due, in large part, to the overexpression of the anti-apoptotic gene product XIAP. The regulation of XIAP overexpression and its transcription and its role in regulating sensitivity to TRAIL has been investigated. We examined the PC-3 cell line as a prototype model system. We have found that constitutive NF-kB activity regulates the resistance to TRAIL and inhibition of NF-kB sensitized the cells to TRAIL-induced apoptosis. The inhibition by NF-kB was due in large part to downregulation of the anti-apoptotic gene products XIAP and Bcl-xL. We also demonstrate that the overexpression of Bcl-xL and XIAP are due in large part to the constitutive activation of NF-kB by tumor derived TNF-a in an autocrine/paracrine manner. The inhibition of NF-kB/ XIAP/ Bcl-xL and sensitization to TRAIL was concomitant with upregulation of DR5. In addition, we demonstrate that overexpression of Bcl-xL and XIAP rendered the cells resistant to CDDP-induced apoptosis and their inhibition sensitizes cells to CDDP apoptosis.

14. SUBJECT TERMS
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TABLE OF CONTENTS

INTRODUCTION

The failure to eradicate advanced prostate cancer that is resistant to conventional therapies, such as chemo and hormonal therapies, has led to the exploration of novel therapeutic applications such as immunotherapy. One form of immunotherapy is to generate anti-tumor cytotoxic lymphocytes that can recognize and eradicate resistant tumor cells. Cytotoxic lymphocytes mediate their killing by various mechanisms including the perforin granzyme pathway and by members of the TNF-α superfamily. Among the TNF-α superfamily, TRAIL has been shown to be selectively cytotoxic to cancer cells and poorly cytotoxic to normal cells and is, therefore, considered as a good candidate for prostate cancer therapy. However, the development of drug/hormonal resistant prostate cancer results in the development of tumor cells resistant to immune killer cells and, indeed, prostate cancer cell lines have been shown to be relatively resistant to TRAIL-induced apoptosis. Resistance is under the regulation of apoptotic regulatory gene products in the cancer cells. We have demonstrated that prostate cancer cells resistant to TRAIL are due in large part to the overexpression of the anti-apoptotic gene product, XIAP and Bcl-$_{xL}$, both of which are under the regulation of constitutive NF-κB. Inhibition of NF-κB or either XIAP or Bcl-$_{xL}$ expression results in the reversal of resistant tumor cells and sensitivity to TRAIL-induced apoptosis. This proposal investigates the role of XIAP in resistance to TRAIL and investigates the underlying mechanisms of the regulation of XIAP expression in prostate cancer cells. We have proposed to investigate 1) The role of XIAP in protecting prostate cancer cells from TRAIL-mediated apoptosis, 2) The regulation of XIAP by NF-κB and NF-κB regulation by XIAP, and 3) The role of endogenous TNF-α and IL-6 in the regulation of XIAP and resistance of tumor cells to TRAIL-induced apoptosis.

BODY

We have made significant progress and novel findings related to the proposed study during the second year.

1) Role of constitutive NF-κB activity and downstream anti-apoptotic gene expression (XIAP and Bcl-$_{xL}$) in the regulation of TRAIL resistance.

We have reported previously that prostate tumor cells are resistant to TRAIL-induced apoptosis. The mechanism underlying resistance was explored and we have reported that overexpression of XIAP is involved in the resistance (Zisman *et al.,* 2001; Ng *et al.,* 2002). We and others have also demonstrated that prostate cancer cell lines exhibit constitutively active nuclear factor kappa B (NF-κB) (Suh *et al.,* 2002; Huerta-Yepez *et al.,* 2004). NF-κB regulates the transcription of many anti-apoptotic gene products, including XIAP and Bcl-$_{xL}$. We examined the role and mechanism of NF-κB-induced resistance to TRAIL apoptosis. We used the nitric oxide donor DETANONOate and the NF-κB inhibitior Bay 11-7085 to inhibit NF-κB activity, and treated PC-3 cells resulted in downstream inhibition of both XIAP and Bcl-$_{xL}$ expression (Figure 1). The inhibition of NF-κB resulted in sensitization to TRAIL apoptosis. Further, the role of Bcl-$_{xL}$ in the regulation of TRAIL resistance was corroborated by the use of the chemical inhibitor 2-methoxyantimycin A which sensitized PC-3 cells to TRAIL-induced apoptosis. We further examined the apoptotic-signaling pathways following treatment of PC-3 cells with the combination of NF-κB inhibitors and TRAIL, and demonstrate that the combination, but not single agents alone, activate the mitochondrial pathway and the activation of caspases 9 and 3 and the induction of apoptosis. The above findings have been recently reported (Huerta-Yepez *et al.,* 2004; Appendix 1).

2) Role of tumor-derived TNF-α in the constitutive activation of NF-κB in PC-3 cells and regulation of TRAIL resistance.

We have previously reported that PC-3 cells synthesize and secrete TNF-α, and TNF-α is a resistant factor (Borsellino *et al.,* 1995). Since TNF-α is a major inducer of NF-κB activity, we hypothesized that tumor-derived TNF-α may activate NF-κB in PC-3 cells via an autocrine/ paracrine loop. The activation of NF-κB by TNF-α will result in the activiation of the anti-apoptotic gene products regulated by NF-κB, such as Bcl-$_{xL}$ and XIAP. The expression of these gene products will then maintain the resistance of the tumor cells to TRAIL-induced apoptosis. This hypothesis was tested experimentally and validated. We demonstrate that treatment of PC-3 cells with exogenous TNF-α activates NF-κB in PC-3 cells (Figure 2A). This finding was confirmed by the use of the NF-κB inhibitor, Bay 11-7085 (Figure 2B). The role on endogenous TNF-α in the regulation of NF-κB activity was demonstrated in studies in which th ePC-3 tumor cells were treated with recombinant soluble TNFR1 (sTNFR1) to neutralize the secreted TNF-α, and inhibits its signaling and activation of NF-κB. The finding demonstrates that such treatment resulted in significant inhibition of NF-κB activity (Figure 2C). These findings suggested that TNF-α secreted by PC-3 cells play a major role in the constitutive activation of NF-kB and

resistant to TRAIL via expression of XIAP and Bcl-$_{xL}$. *In vivo* prostate cancer cells that do not secrete TNF-α may be influenced by TNF-α-derived from the microenvironment to regulate their resistance to TRAIL-apoptosis. The above studies and additional studies are being completed for publication.

3) Mechanism of NF-κB mediated regulation of TRAIL resistance: Suppression of DR5 transcription

We examined the potential mechanism by which NF-κB activation, aside from the expression of XIAP and Bcl-$_{xL}$, regulates PC-3 cells' resistance to TRAIL. We have found that inhibitors of NF-κB, which induced sensitization of PC-3 to TRAIL induced apoptosis, correlated with the upregulation of the TRAIL receptor DR5. The upregulation of DR5 was determined by flow, RT-PCR and western (Figure 3). We then examined the upregulation of DR5 expression using a luciferase reporter system that we have obtained from our collaborator Dr. Sakai in Kyoto, Japan (Yoshida *et al.,* 2001). We analyzed constructs and demonstrate that NF-κB inhibitors such as DHMEQ (Ariga *et al.,* 2002) significantly augmented luciferase activity and confirmed the above findings (Figure 4). We are currently examining several other constructs of the reporter system developed by Dr. Sakai in order to identify NF-κB regulated transcription factors that modulate negatively the transcription of DR5. One putative transcription factor is the transcription repressor Yin Yang 1 (YY1) that we have previously reported to negatively regulate the transcription of the Fas receptor (Garban and Bonavida, 2001).

4) Role of XIAP and Bcl-$_{xL}$ expression in the regulation of PC-3 resistance to CDDP-induced apoptosis

We have found that prostate cancer tumor cell lines (PC-3, CL-1, LNCaP) are resistant to CDDP-mediated apoptosis. We examined whether the resistance is due in part to the constitutive activation of NF-κB and downstream regulation of XIAP and Bcl-$_{xL}$ expression similar to the resistance observed against TRAIL. We also hypothesized that tumor-derived cytokines (e.g. TNF-a) that regulates the constitutive activity of NF-κB and downstream anti-apoptotic gene products like XIAP and Bcl-$_{xL}$ will result in the regulation of tumor cell resistance to CDDP. Hence, interfering with this pathway should sensitize the cells to CDDP apoptosis (Figure 5). This hypothesis was tested and verified experimentally. We have found that inhibition of endogenous TNF-α by recombinant sTNFR1 sensitizes PC-3 cells to CDDP-induced apoptosis (Figure 5). Further, inhibition of NF-κB by Bay 11-7085 mimicked the neutralization of TNF-α and sensitized the cells to CDDP apoptosis (Figure 6). As shown previously in Figure 1, the inhibition of NF-κB resulted in the inhibition of XIAP and Bcl-$_{xL}$. We demonstrate that inhibition of Bcl-$_{xL}$ by the inhibitor 2-methoxyantimycin A sensitizes cells to CDDP-induced apoptosis. The direct role of XIAP in the inhibition of CDDP-induced apoptosis was examined by the use of actinomycin D which we have earlier reported selectively inhibits XIAP expression (Ng and Bonavida, 2002). Treatment of PC-3 with Act D resulted in sensitization of PC-3 cells to CDDP-induced apoptosis (Figure 7). These findings demonstrate that NF-κB and gene products XIAP and Bcl-$_{xL}$ regulate the resistance of PC-3 cells to CDDP-induced apoptosis. These studies and others in progress will be completed for

publication. The above preliminary findings were presented at a mini-symposium in the 2004 AACR meeting in Orlando, Florida. A copy of the abstract is enclosed as Appendix 2.

5) Expression of XIAP in prostate tumor tissue microarrays

We have completed the staining and specificity of XIAP expression in prostate tumor tissue microarrays. Case materials from 246 prostatectomies were arrayed into 3 blocks encompassing a total of 1,364 individual tissue cases. A standard 2-step indirect avidin/biotin complex (ABC method was used). Included in the study PIN, BPH, and normal tissues from the same pool were analyzed. In addition, the frequency of positive target cells (Range 0-100%) will be scored for each tissue microarray spot. Statistical analysis will examine the association between XIAP expression and clinical pathological variables using the Pearson chi-square test. Kaplan-Meier and Cox analyses will be performed by Dr. Steve Horvath, a biostatistician in the Department of Human Genetics. We anticipate the analysis to be completed shortly.

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KEY RESEARCH ACCOMPLISHMENTS

1. We have completed studies on the expression of XIAP on prostate cancer tissue microarrays and analyses are currently being performed.

2. We have demonstrated that prostate cancer cell lines express constitutively, activated NF-κB. NF-κB regulates the transcription of XIAP and Bcl-$_{xL}$ and inhibition of NF-κB downregulates their expression and sensitizes the cells to TRAIL-induced apoptosis.

3. We have demonstrated that PC-3 cells secrete TNF-α which acts by autocrine/paracrine fashion as an activator of NF-κB. Inhibition of TNF-α by anti-TNF-α antibody or by soluble TNFR-1 downregulates NF-κB activity and the expression of XIAP and Bcl-$_{xL}$ and sensitizes the cells to TRAIL-induced apoptosis.

4. We have demonstrated that NF-κB negatively regulates the expression of the TRAIL receptor, DR5. Inhibition of NF-κB by chemical inhibitors or by nitric oxide donor (which S-nitrosylates p50) resulted in the upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. These findings were corroborated in PC-3 cells transfected with the DR5 promoter and treatment with NF-κB inhibitors or by nitric oxide resulted in significant increase in the luciferase activity.

5. We have also found that NF-κB regulates Bcl-$_{xL}$ expression in PC-3 cells. Inhibition of Bcl-$_{xL}$ activity results in sensitization of the cells to TRAIL-mediated apoptosis. These findings reveal that in PC-3, both XIAP and Bcl-$_{xL}$ overexpression regulates the sensitivity of prostate cancer cells to TRAIL apoptosis.

6. We have found that NF-κB also regulates Bcl-xL expression in prostate cancer cells and both XIAP and Bcl-$_{xL}$ overexpression render the cells resistant to chemotherapeutic drugs-induced apoptosis. This finding suggests that there is cross-resistance between immune-mediated and drug-induced apoptosis.

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1. Huerta-Yepez S, Vega M, Jazirehi A, Garban H, Hongo F, Cheng G, and Bonavida B. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kB and inhibition of Bcl-xL expression. *Oncogene*, 23: 4993-5003, 2004.

2. Huerta-Yepez S, Vega M, Hongo F, Jazirehi A, Garban H, Mizutani Y, and Bonavida B. Regulation of prostate cancer sensitivity to apoptosis by CDDP by downregulation of XIAP and Bcl-$_{xL}$ expression via inhibition of constitutive NF-κB activity. Abstract No. 4826, AACR March 2004 Annual Meeting- Manuscript in Preparation.

3. Huerta-Yepez S, Vega M, Escoto-Chavez SE, Murdock B, Sakai T, and Bonavida B. Inhibition of DR5 expression and regulation of TRAIL resistance in cancer cells by the transcription repressor YY1. (Manuscript in preparation).

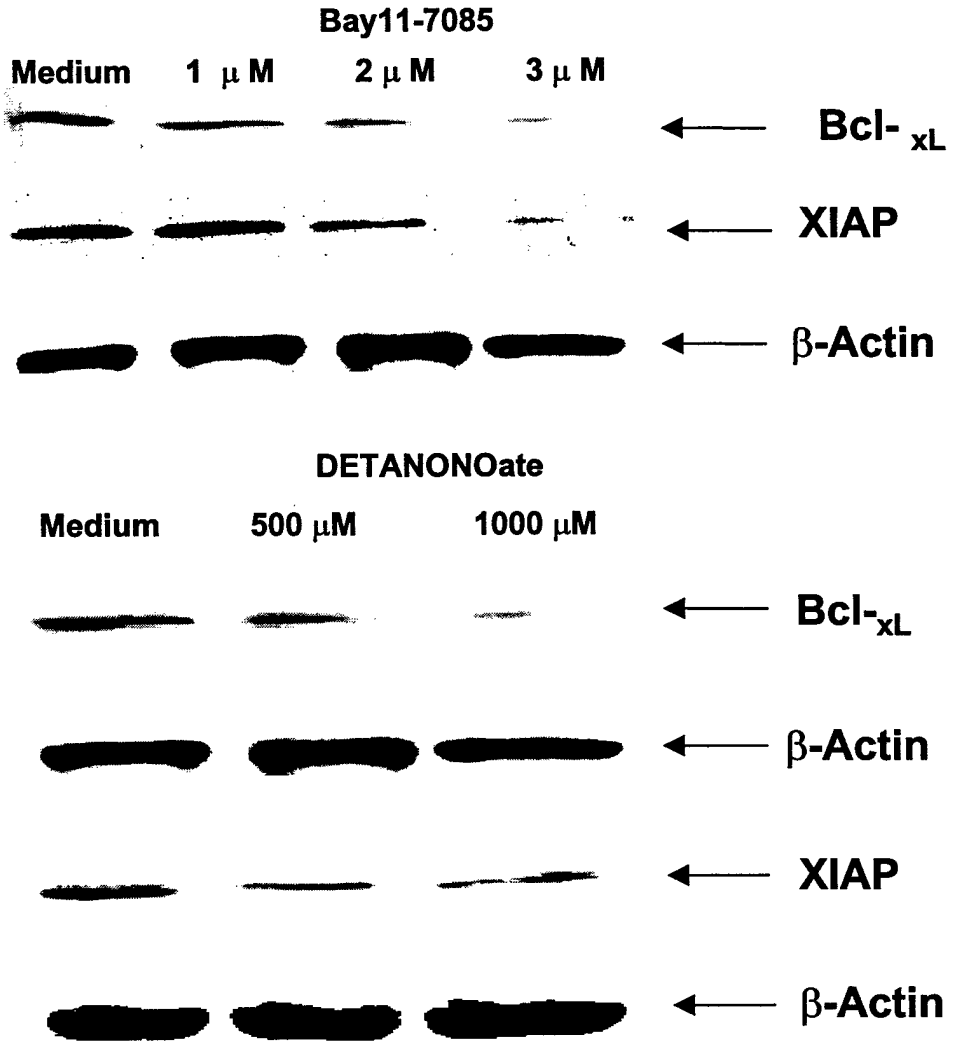
CONCLUSIONS

Our findings on the regulation of XIAP expression in CaP cells have revealed that XIAP is in part under the transcriptional regulation of constitutive NF-κB. Constitutive NF-κB is under the control of tumor-derived TNF-α by an autocrine/paracrine mechanism. This loop, therefore, regulates the survival and resistance of CaP to TRAIL-induced apoptosis by overexpression of XIAP. In addition to XIAP, our new findings revealed that NF-κB regulates another anti-apoptotic gene product, Bcl-$_{xL}$, which is also under the transcriptional regulation of NF-κB and also regulates the resistance to TRAIL. In our studies, we have observed an unexpected finding demonstrating that NF-κB negatively regulates the expression of the TRAIL receptor, DR5. The mechanism by which NF-κB regulates DR5 is not known. We have postulated that NF-κB may be regulating another transcription factor, a transcriptional repressor that is present in the DR5 promoter. We have postulated that the transcriptional repressor, Yin-Yang 1 (YY1), may be involved in the negative regulation of DR5 expression. We have evidence demonstrating that inhibition of YY1 results in the upregulation of DR5 and sensitization to TRAIL as well as evidence demonstrating that YY1 is under the transcriptional regulation of NF-κB. Our current studies are examining the role of YY1 in the regulation of DR5 using DR5 reporter systems lacking the YY1 binding sites. Our other studies using prostate cancer tissue microarrays have revealed that cancerous tissue show overexpression of XIAP in the cytoplasm and we are currently completing the analyses and anticipate its clinical prognostic significance. Overall, our findings have corroborated the hypotheses suggested in the proposal and we have also made novel observations that we contemplate completing during the coming year in addition to the unfinished studies.

REPORTABLE OUTCOMES

We have published one manuscript and we are in the process of completing two additional manuscripts for submission.

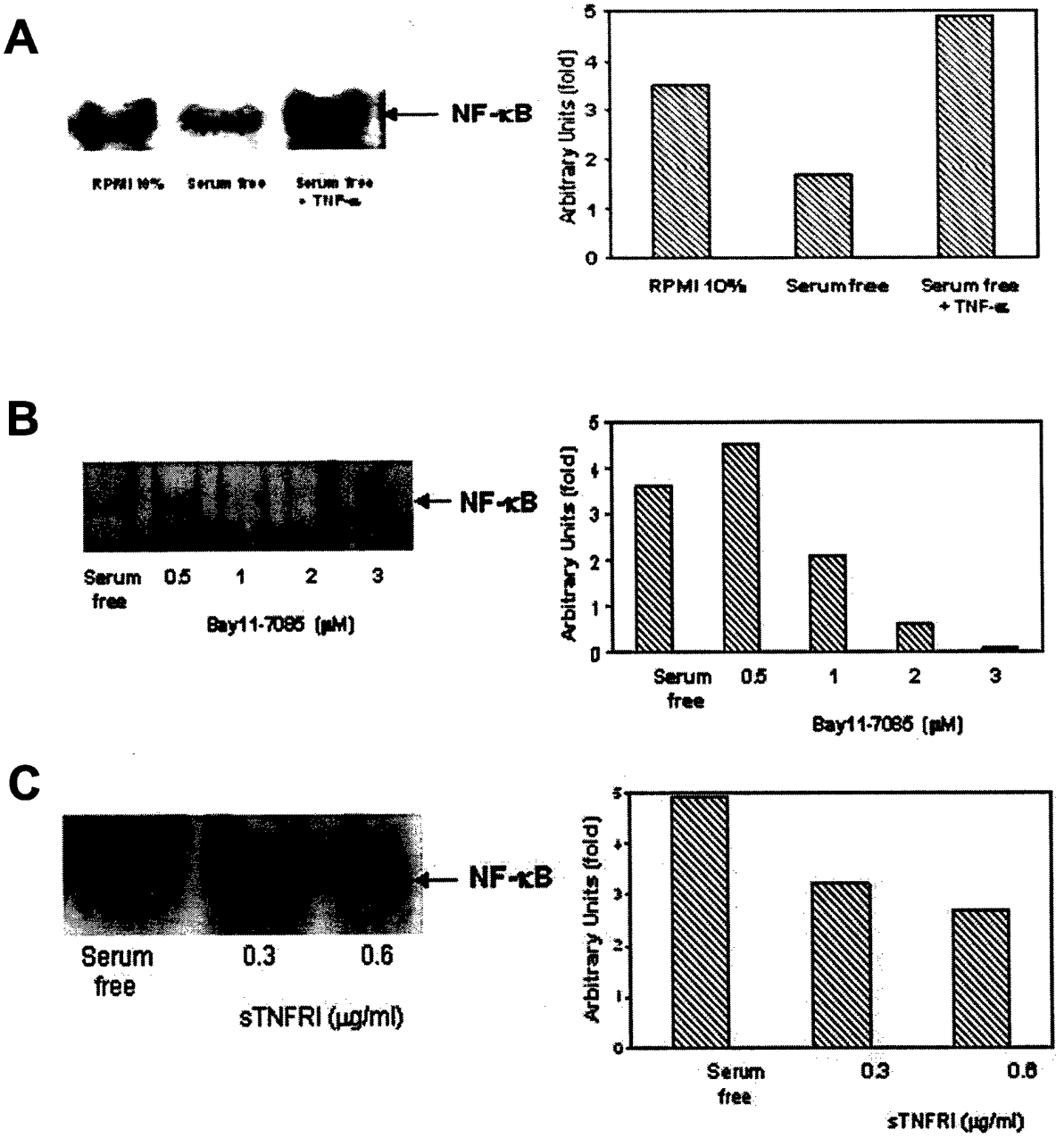
Inhibition of NF-κB induces downregulation of Bcl-$_{xL}$ and XIAP expression



PC-3 tumor cells were treated with different concentrations of the NF-κB inhibitors Bay 11-7085 (top) and DETANONOate (bottom) for 18 h and cell lysates were examined by western for the expression of Bcl-$_{xL}$ and XIAP. The data demonstrates that both Bay 11-7085 and DETANONOate inhibits both Bcl-$_{xL}$ and XIAP expression, and the extent of inhibition was dependent on the concentrations used.

Figure 1

Regulation of NF-κB by TNF-α

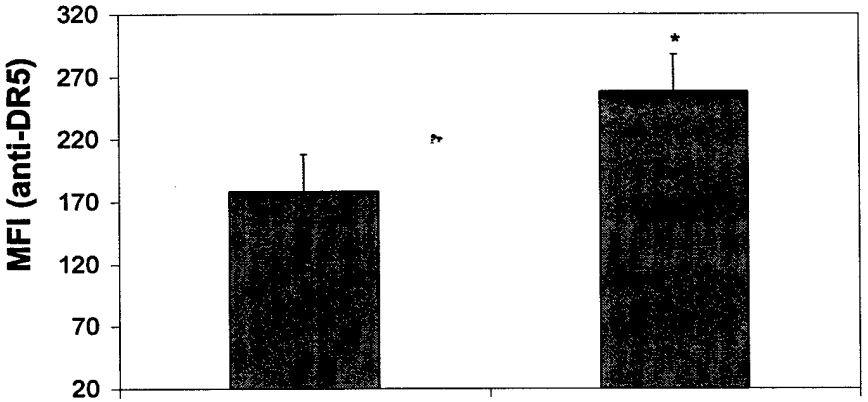


A) PC-3 cells were cultured in RPMI with 10% FBS or serum free. The serum free cultures were treated with TNF-α (A) or various concentrations of the NF-κB inhibitor Bay 11-7085 (B) or with two different concentrations of recombinant sTNFR1 (C) for 18 h. The cells were then harvested and nuclear lysates prepared and tested for NF-κB DNA-binding activity by EMSA. The finding demonstrates that exogenous TNF-α activates NF-κB whereas the NF-κB inhibitor or sTNFR1 significantly inhibited NF-κB activity.

Figure 2

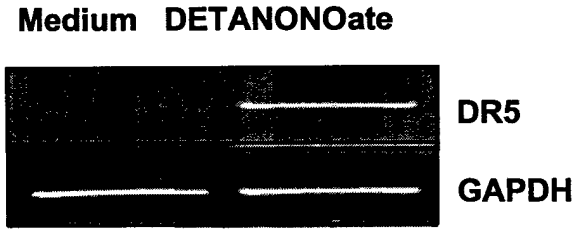
Upregulation of DR5 expression by inhibition of NF-κB activity

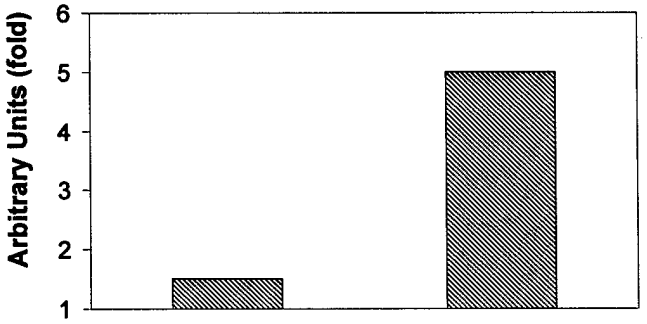
A



PC-3 cells were treated with 1000 μM of DETANONOate for 18 h and DR5 surface expression was performed by flow (A) , RT-PCR (B) and by western (C). It is clear that the NF-κB inhibitor DETANONOate upregulates surface DR5 expression, DR5 transcription and protein expression.

B





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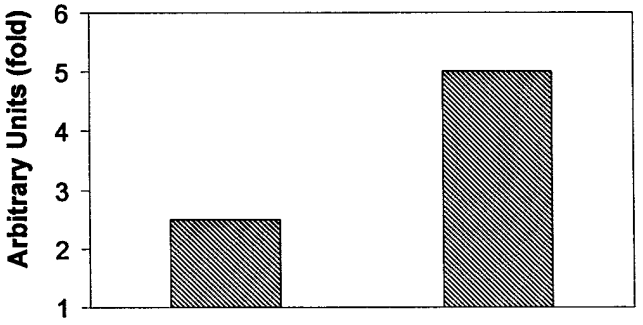
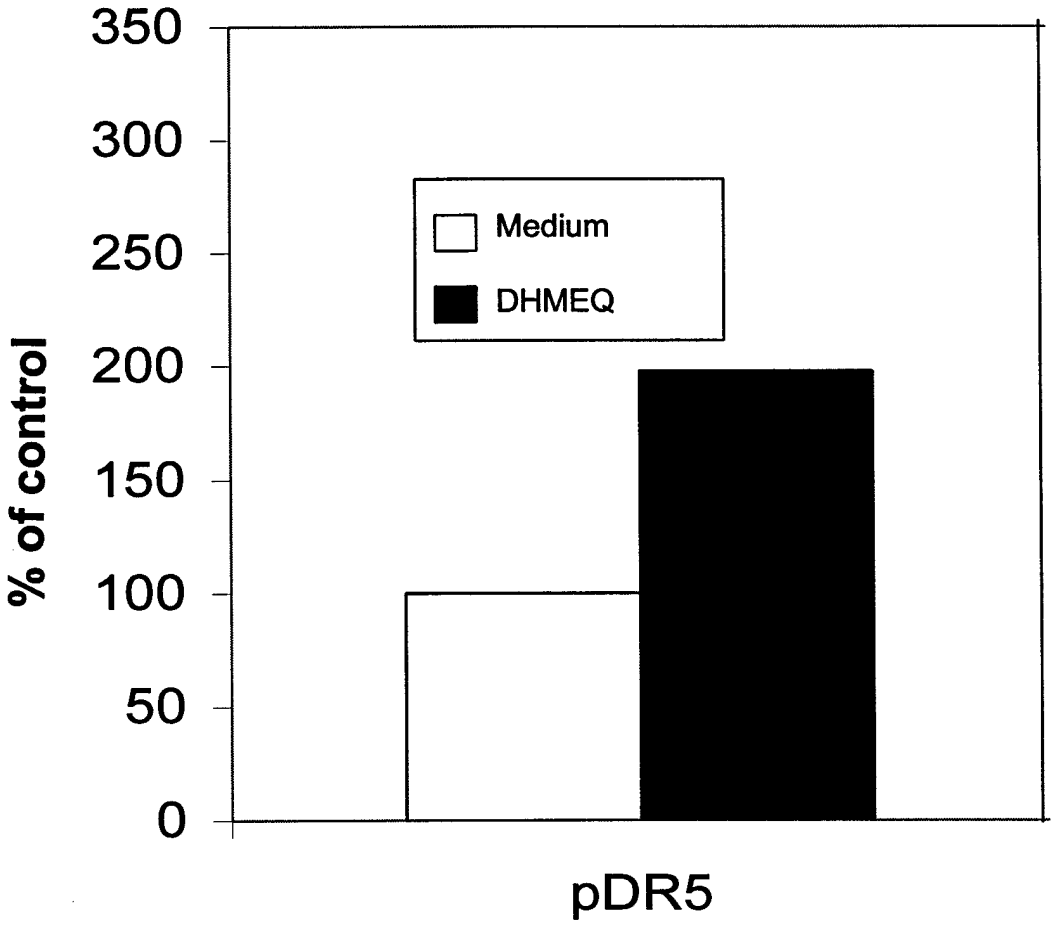


Figure 3

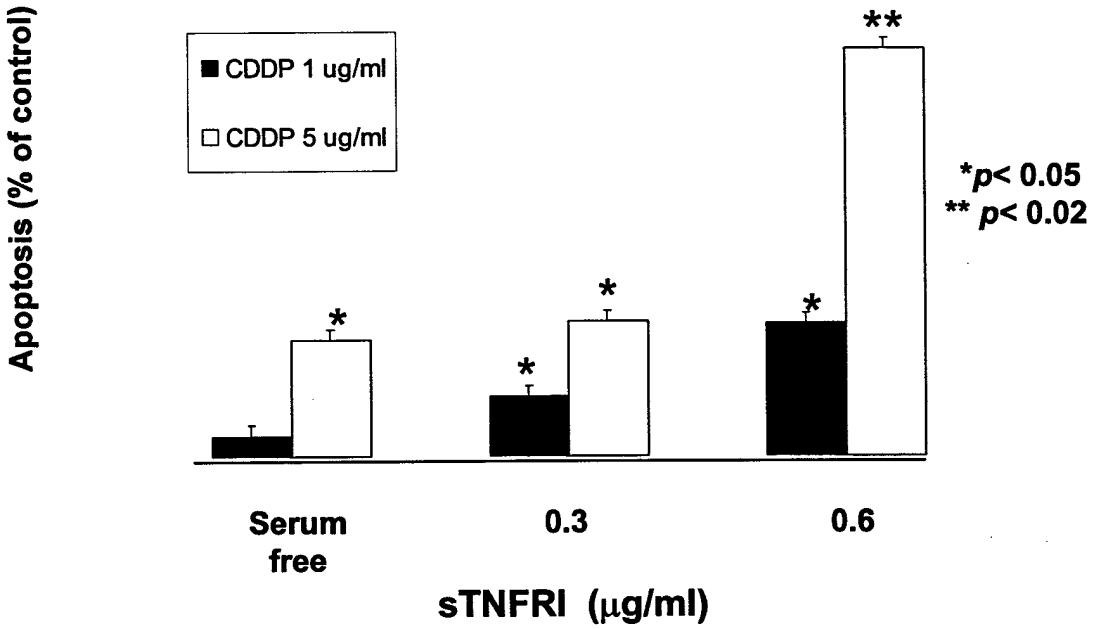
Upregulation of DR5 by the NF-κB inhibitor DHMEQ



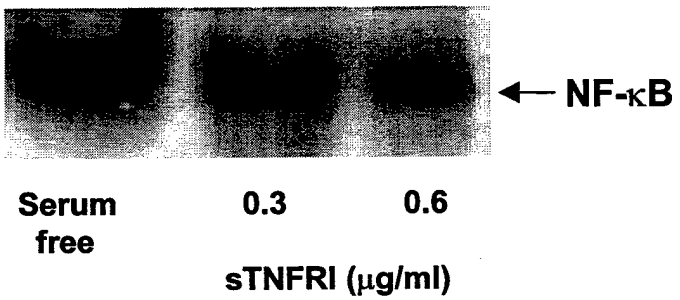
PC-3 tumor cells were transfected with the pDR5 luciferase reporter system, and a sample was treated with 10 μg/ml DHMEQ. Luciferase activity was measured. The data demonstrate that the NF-κB inhibitor upregulated DR5 transcription.

Figure 4

Inhibition of endogenous TNF-α inhibits NF-κB and sensitizes PC-3 cells to CDDP-mediated apoptosis



PC-3 cells were treated with CDDP in the presence or absence of sTNFR1 for 18 h and apoptosis was determined by activation of caspase 3. The data demonstrate that neutralization of endogenous TNF-α sensitizes cells to CDDP-induced apoptosis. This correlated with inhibition of NF-κB activity as determined by EMSA.



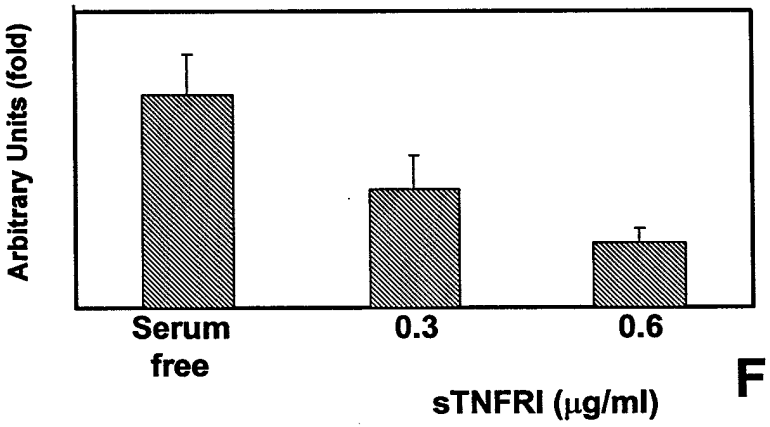
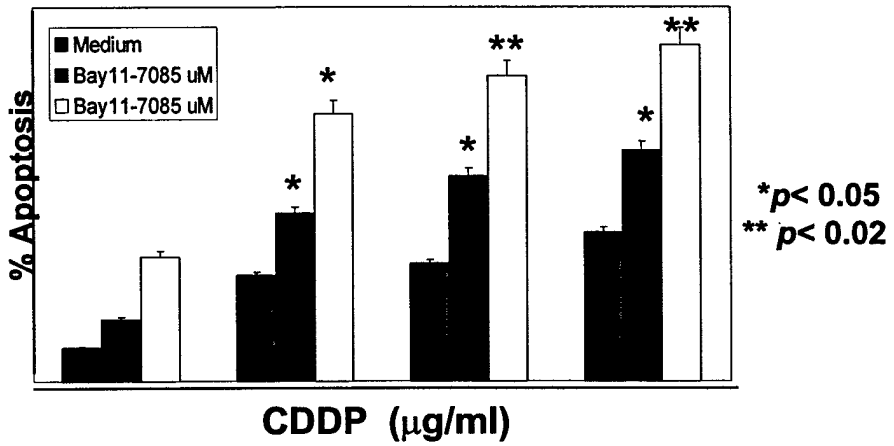


Figure 5

Bay 11-7085 inhibitor of NF-κB sensitizes PC-3 cells to CDDP-mediated apoptosis

Apoptosis



PC-3 cells were treated with the NF-κB inhibitor Bay 11-7085 in the presence or absence of CDDP for 18 h, and apoptosis was measured by activation of caspase 3. The data demonstrates that Bay 11-7085 significantly sensitizes cells to CDDP-mediated apoptosis (Top). This was correlated with Bay 11-7085-induced inhibition of NF-κB activity (Bottom).

EMSA

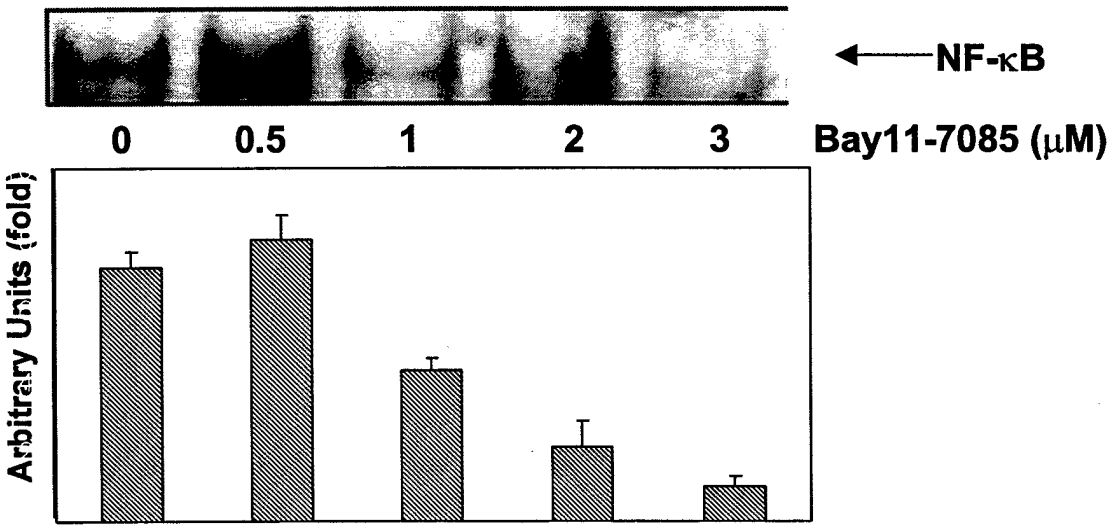
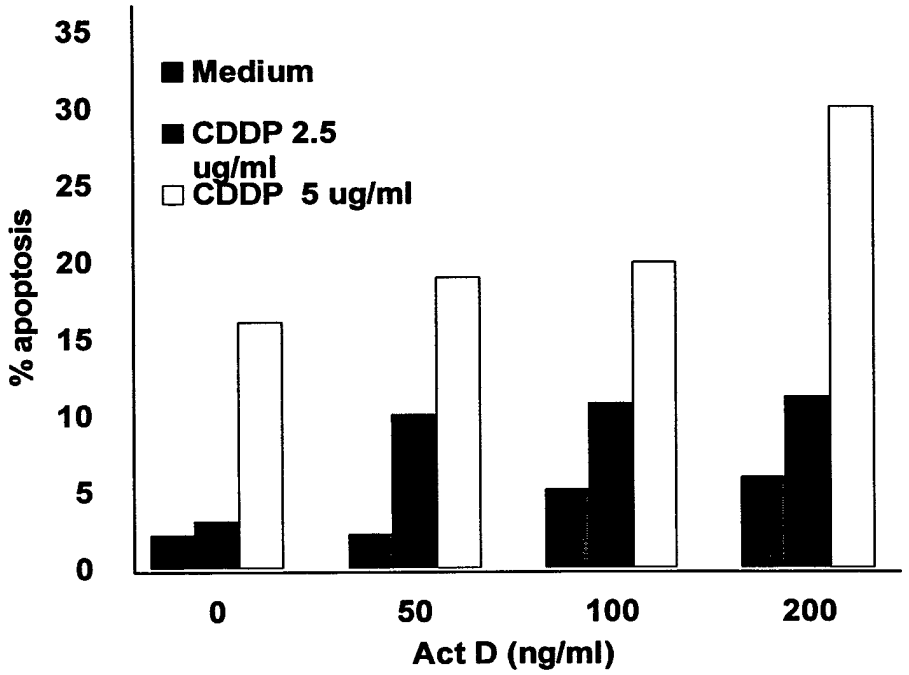
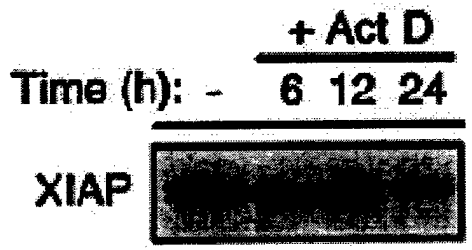


Figure 6

Actinomycin D selectively induces downregulation of XIAP expression and sensitizes PC-3 cells to CDDP-mediated apoptosis.



PC-3 cells were treated with actinomycin D in the presence or absence of CDDP for 18 h, and apoptosis was calculated (Top), and XIAP expression by western (Bottom). The data demonstrates that actinomycin D significantly sensitized PC-3 cells to CDDP-induced apoptosis. There was a correlation between downregulation of XIAP and sensitization.



Act D inhibits XIAP expression (Ng *et al.* Prostate, 53:286-299. 2002)

Figure 7

Oncogene (2004) 23, 4993–5003

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Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-κB and inhibition of Bcl-$_{xL}$ expression

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues and this prompted its potential therapeutic application in cancer. However, not all cancers are sensitive to TRAIL-mediated apoptosis and, therefore, TRAIL-resistant cancer cells must be sensitized first to become sensitive to TRAIL. Treatment of prostate cancer (CaP) cell lines (DU145, PC-3, CL-1, and LNCaP) with nitric oxide donors (e.g. (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate (DETANONOate)) sensitized CaP cells to TRAIL-induced apoptosis and synergy was achieved. The mechanism by which DETANONOate mediated the sensitization was examined. DETANONOate inhibited the constitutive NF-κB activity as assessed by EMSA. Also, p50 was S-nitrosylated by DETANONOate resulting in inhibition of NF-κB. Inhibition of NF-κB activity by the chemical inhibitor Bay 11-7085, like DETANONOate, sensitized CaP to TRAIL apoptosis. In addition, DETANONOate downregulated the expression of Bcl-2 related gene (Bcl-$_{xL}$) which is under the transcriptional regulation of NF-κB. The regulation of NF-κB and Bcl-$_{xL}$ by DETANONOate was corroborated by the use of Bcl-$_{xL}$ and Bcl-x κB reporter systems. DETANONOate inhibited luciferase activity in the wild type and had no effect on the mutant cells. Inhibition of NF-κB resulted in downregulation of Bcl-$_{xL}$ expression and sensitized CaP to TRAIL-induced apoptosis. The role of Bcl-$_{xL}$ in the regulation of TRAIL apoptosis was corroborated by inhibiting Bcl-$_{xL}$ function by the chemical inhibitor 2-methoxyantimycin A_3 and this resulted in sensitization of the cells to TRAIL apoptosis. Signaling by DETANONOate and TRAIL for apoptosis was examined. DETANONOate altered the mitochondria by inducing membrane depolarization and releasing modest amounts of cytochrome *c* and Smac/DIABLO in the absence of downstream activation of caspases 9 and 3. However, the combination of DETANONOate and TRAIL resulted in activation of the mitochondrial pathway and activation of caspases 9 and 3, and induction of apoptosis. These findings demonstrate that DETANONOate-mediated sensitization of CaP to TRAIL-induced apoptosis is via inhibition of constitutive NF-κB activity and Bcl-$_{xL}$ expression.

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Introduction

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutics such as drugs, irradiation, and immunotherapy since most of their primary cytotoxic effects are through apoptosis (Ng and Bonavida, 2002a; Hersey and Zhang, 2003). Therefore, after the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, antitumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (Ashkenazi and Dixit, 1999; Ashkenazi *et al.*, 1999). TRAIL has been considered a new therapeutic, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (Ashkenazi *et al.*, 1999; De Jong *et al.*, 2001; Wajant *et al.*, 2002; Chawla-Sarkar *et al.*, 2003). However, many tumor cells have been shown to be resistant to TRAIL (Zisman *et al.*, 2001; Ng *et al.*, 2002; Bouralexis *et al.*, 2003; Tillman *et al.*, 2003). We and others have reported that various sensitizing agents like chemotherapeutic drugs (Zisman *et al.*, 2001; Munshi *et al.*, 2002), cytokines (Park *et al.*, 2002), and inhibitors (Nyormoi *et al.*, 2003) are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

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Prostate cancer (CaP) cells have been shown to exhibit constitutive nuclear factor κB (NF-κB) activity (Suh *et al.*, 2002). It has been recently reported that NF-κB can regulate the sensitivity of target cells to TRAIL apoptosis in hepatoma cells (Shigero *et al.*, 2003). In addition, it has been reported that CaP cells overexpress Bcl-2 related gene (Bcl-$_{xL}$), which negatively regulates tumor cells sensitivity to drug-mediated apoptosis (Raffo *et al.*, 1995). Studies on Bcl-$_{xL}$ gene transcription demonstrate that Bcl-$_{xL}$ is regulated in part by NF-κB (Mori *et al.*, 2001). Thus, constitutive expression of NF-κB in CaP may regulate the constitutive expression of Bcl-$_{xL}$. We have reported that nitric oxide (NO) donors can sensitize tumor cells to FasL and tumor necrosis factor alpha (TNF-α)-mediated apoptosis (Garban and Bonavida, 2001a, b). Further, we (Huerta-Yepez *et al.*, 2003) and others (Lee *et al.*, 2001; Secchiero *et al.*, 2001) reported that (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1, 2-diolate (DETANONOate) can also sensitize tumor cells to TRAIL-mediated apoptosis.

The mechanism underlying the NO-mediated sensitization to TRAIL is not known. We hypothesized that NO-mediated sensitization of CaP cells to apoptosis may be due to NO-induced inhibition of constitutive NF-κB activity and this, in turn, will result in the downregulation of Bcl-$_{xL}$ transcription and expression. Hence, downregulation of the antiapoptotic gene product Bcl-$_{xL}$ will result in the sensitization of CaP cells to TRAIL-mediated apoptosis. This study was designed to test this hypothesis and the followings were investigated: (1) Does NO sensitize androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis? (2) Does NO inhibit constitutive NF-κB activity resulting in inhibition of Bcl-$_{xL}$ expression? (3) Do inhibitors of NF-κB and Bcl-$_{xL}$ mimic NO and sensitize CaP to TRAIL-mediated apoptosis? And (4) by what mechanism does NO modify the apoptotic signaling pathway and sensitize CaP to TRAIL-mediated apoptosis?

Results

Sensitization of CaP cell lines to TRAIL-mediated apoptosis by DETANONOate

Our previous findings have demonstrated that CaP cell lines (LNCaP, DU-145, PC-3, and CL-1) are relatively resistant to TRAIL-mediated apoptosis (Zisman *et al.*, 2001; Ng *et al.*, 2002), and are shown in Figure 1a. However, pretreatment of CaP cell lines with the NO donor DETANONOate resulted in significant potentiation of apoptosis by TRAIL for the four cell lines tested. The extent of potentiation was a function of the concentration of TRAIL used (Figure 1a). The sensitization by DETANONOate was synergistic as determined by isobologram analysis (Figure 1b). We selected PC-3 as a model system for further investigation. Treatment of PC-3 cells with various concentrations of DETANONOate sensitized the cells to TRAIL-induced apoptosis, and the extent of apoptosis was a function of the concentration of DETANONOate used (Figure 1c). In addition to apoptosis, NO, TRAIL, and the combination inhibited cell proliferation significantly (Figure 1d). These findings demonstrate that DETANONOate sensitizes androgen-dependent and -independent CaP tumor cell lines to TRAIL-mediated apoptosis and synergy is achieved. Previous findings demonstrated that the androgen 5-α dihydrotestosterone (DHT) sensitizes LNCaP to 12-*O*-tetradecanoylphorbolacetate (TPA)-induced apoptosis (Altuwaijri *et al.*, 2003). We examined whether DHT also sensitizes LNCaP to TRAIL apoptosis. We observed that treatment of LNCaP with DHT sensitizes the cells to TRAIL (Table 1).

DETANONOate inhibits NF-κB activity and inhibition of NF-κB sensitizes PC-3 to TRAIL apoptosis

We examined the effect of DETANONOate on NF-κB activity in PC-3 cells. The cells were treated with DETANONOate (500 and 1000 μM) and tested for NF-κB activity by EMSA. In addition, we used the NF-κB inhibitor, Bay 11-7085, at different concentrations as control for inhibition of NF-κB activity. Figure 2a demonstrates that DETANONOate inhibits NF-κB activity significantly and the inhibition at 1000 μM was much higher than the inhibition at 500 μM. As expected, the Bay 11-7085 inhibitor also significantly inhibited NF-κB activity, and the inhibition was a function of the concentration of Bay 11-7085 used (Figure 2a).

It has been reported that the DNA-binding activity of NF-κB p50 can be modified by NO and p50 becomes S-nitrosylated and inhibits NF-κB activity (Matthews *et al.*, 1996; Dela Torre *et al.*, 1997; Marshall and Stamler, 2001). Thus, we examined whether DETANONOate treatment of PC-3 cells induces S-nitrosylation of p50. PC-3 cells were grown in the absence or presence of DETANONOate (500 or 1000 μM) for 18 h and total cell lysates were prepared and immunoprecipitation assay was performed as described in Materials and methods. Using anti-S-nitrosylated antibody, the S-nitrosylated proteins were immunoprecipitated and were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with

Table 1 DHT sensitizes LNCaP to TRAIL-mediated apoptosis

	TRAIL (ng/ml)		
DHT (nM)	*0*	*5*	*10*
0	5.1±1	12±2.1	17±3.8
10	6.6±0.9	18±1.1*	24±5.1*
20	6.9±1.1	23±6.1*	30.6±6.3**

LNCaP cells were treated or left untreated with DHT (10 or 20 nM) for 24 h and then treated with recombinant TRAIL (5 or 10 ng/ml) for 18 h. The cells were harvested and apoptosis was determined for cells with active caspase 3 staining by flow. The data show that DHT sensitizes LNCaP to TRAIL-mediated apoptosis. The data represent the mean of two independent experiments. *$P<0.04$, **$P<0.02$ compared with the cells treated with DHT alone

anti-NF-κB p50 antibody. S-nitrosylation of p50 was significantly enhanced following DETANONOate treatment (Figure 2b).

The relationship between DETANONOate-mediated inhibition of NF-κB and sensitization to TRAIL was examined. PC-3 cells were treated with various concentrations of Bay 11-7085 (1–5 μM) and TRAIL (5 and 10 ng/ml). Treatment with Bay 11-7085 significantly potentiated the sensitivity of PC-3 to TRAIL-mediated apoptosis, and the degree of apoptosis was a function of the concentration used (Figure 2c).

These findings demonstrate that DETANONOate inhibits NF-κB activity and results in the sensitization of PC-3 to TRAIL-induced apoptosis. Further, the results suggest that DETANONOate-mediated sensitization is via inactivation of NF-κB.

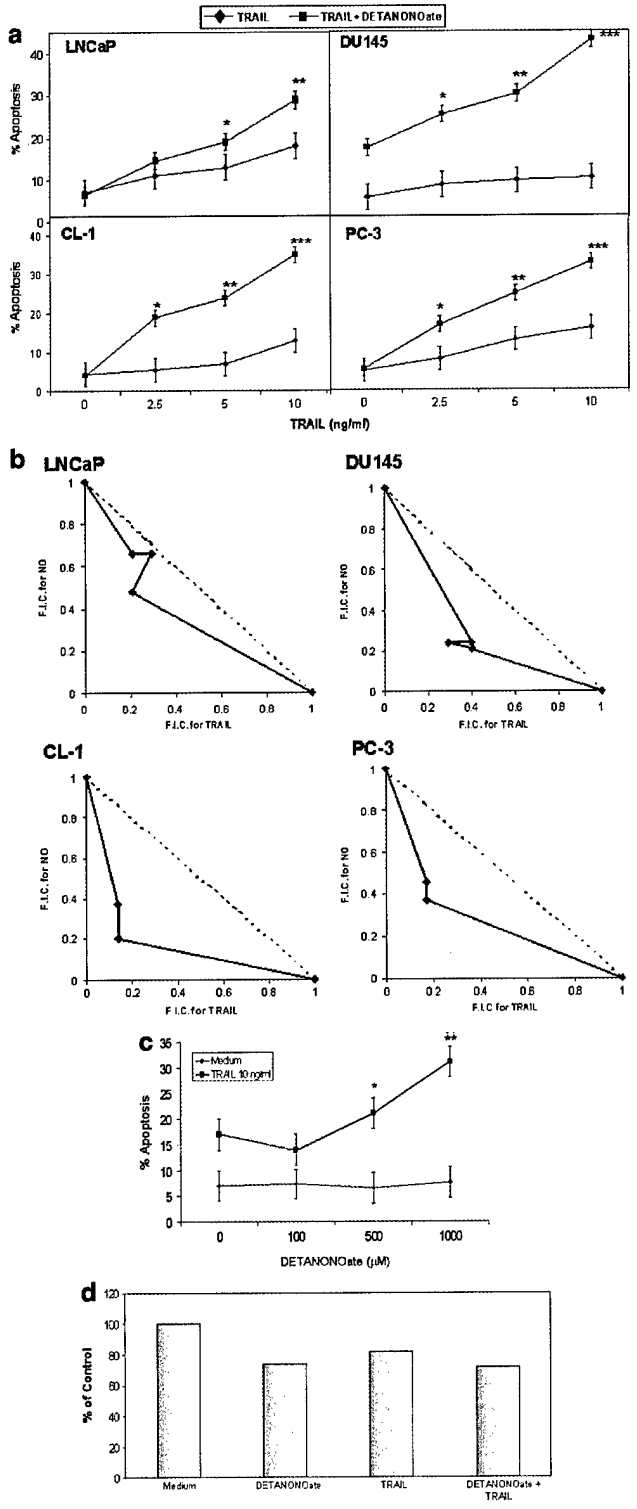

Figure 1 DETANONOate sensitizes CaP cell lines to TRAIL-mediated apoptosis. (**a**) The CaP cell lines DU145, CL-1, and PC-3 were grown in FBS-free medium and LNCaP cells were grown in a medium with 1% FBS. The cell lines were treated with different concentrations of TRAIL (0, 2.5, and 5 ng/ml) in the presence or absence of DETANONOate (1000 μM) for 18 h at 37° in a 5% CO_2 incubator. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analysed by flow cytometry as described in Materials and methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. $*P<0.05$, $**P<0.02$, $***P<0.004$. (**b**) This figure establishes synergy as determined by isobologram analysis. (**c**) PC-3 cells were grown in FBS-free medium and were treated with TRAIL (5 ng/ml) in the presence or absence of different concentrations of DETANONOate (100, 500, and 1000 μM) for 18 h and analysed for apoptosis. Significant sensitization was observed at DETANONOate concentrations of 500 and 1000 μM. (**d**) The PC-3 cells were treated with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), and the combination, and viable cell recovery was examined microscopically by Trypan blue dye exclusion at 24 h. The data show that all agents inhibited cell proliferation

DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression and sensitization to TRAIL

DETANONOate selectively inhibited Bcl-$_{xL}$ expression in PC-3 with little effect on other pro- and antiapoptotic gene products examined (Figure 3a). TRAIL has no effect on any of the gene products examined. It has been reported that Bcl-$_{xL}$ transcription is regulated in part by NF-κB (Mori *et al.*, 2001; Sevilla *et al.*, 2001). Thus, it was possible that DETANONOate-mediated inhibition of NF-κB (Figure 2a) was responsible for the observed DETANONOate-mediated inhibition of Bcl-$_{xL}$ expression (Figure 3a). This was confirmed by demonstrating that treatment of PC-3 with the NF-κB inhibitor Bay 11-7085, like DETANONOate, also inhibited Bcl-$_{xL}$ expression (Figure 3b). Therefore, it was possible that sensitization of PC-3 by DETANONOate to TRAIL-induced apoptosis was due in part to downregulation of Bcl-$_{xL}$ expression via inhibition of NF-κB. Accordingly, inhibition of Bcl-$_{xL}$ expression should sensitize PC-3, like NO, to TRAIL-induced apoptosis. Treatment of PC-3 with the Bcl-$_{xL}$ inhibitor 2-methoxyantimycin A_3 (2MAM-A3) (Tzung *et al.*, 2001) resulted in significant sensitization of the cells to TRAIL-induced apoptosis. The potentiation was a function of the concentration of 2MAM-A3 used (Figure 3c). These findings suggest that Bcl-$_{xL}$ is the dominant resistant factor in PC-3 cells for TRAIL-induced apoptosis, and Bcl-$_{xL}$ inhibition by DETANONOate via NF-κB

inactivation may be responsible for sensitization to TRAIL.

It has been reported that NF-κB activity plays an important role in the transcriptional regulation of Bcl-$_{xL}$ (Mori *et al.*, 2001; Sevilla *et al.*, 2001). To determine whether NF-κB activity is required for Bcl-$_{xL}$ transcription and to determine how DETANONOate induces selective inhibition of Bcl-$_{xL}$ via NF-κB, transient transfection assays were performed. PC-3 cells were transfected with the Bcl-x WT promoter and Bcl-x κB promoter reporter plasmids. At 24 h after transfection, the cells were treated with either Bay 11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or optimal concentrations of TNF-α (50 or 100 U/ml) for 18 h. Both DETANONOate treatment and Bay 11-7085 treatment induced significant inhibition of Bcl-$_{xL}$ transcription, and the extent of inhibition was concentration dependent. In contrast, activation of NF-κB by TNF-α treatment induced a significant increase in Bcl-$_{xL}$ transcription (Figure 4). The basal luciferase activity was significantly reduced in the mutant (5×) compared to wild type, suggesting that Bcl-$_{xL}$ transcription in PC-3 is primarily regulated by NF-κB. In contrast to the findings in the wild type, the different treatments did not affect the cells transfected with the Bcl-x κB promoter (Figure 4). These results indicate that Bcl-$_{xL}$ transcription in PC-3 is in large part regulated by NF-κB, and inhibition of NF-κB by DETANONOate is responsible for DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression.

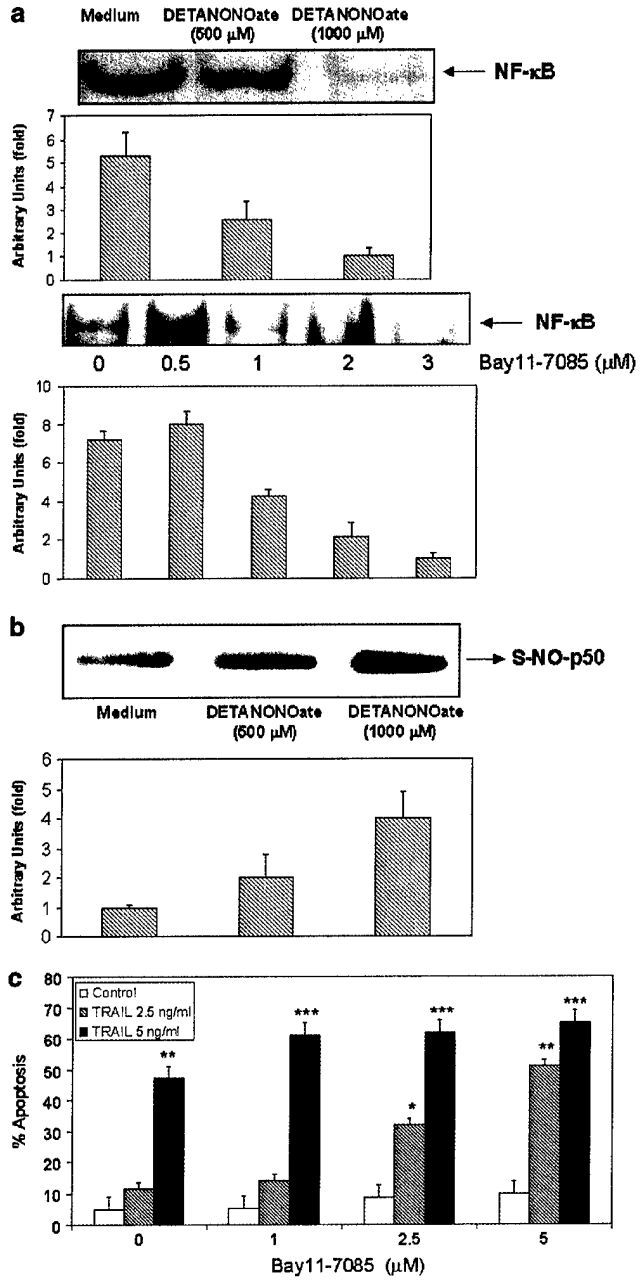

Figure 2 NF-κB is involved in TRAIL-mediated apoptosis in PC-3 cells. (**a**) Inhibition of NF-κB activity. Nuclear extracts from PC-3 cells grown in FBS-free medium were treated or left untreated with DETANONOate (500 or 1000 μM) (top panel), or treated with different concentrations of the specific NF-κB inhibitor Bay 11-7085 (0, 0.5, 1, 2, and 3 μM) (bottom panel), and were analysed by EMSA to assess NF-κB DNA-binding activity. Relative NF-κB binding activity was determined by densitometry analysis. The findings demonstrate that treatment of PC-3 cells with DETANONOate results in inhibition of NF-κB activity. (**b**) Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50) upon DETANONOate (500 and 1000 μM, 18 h) treatment. Total cell lysates were used in an immunoprecipitation assay using protein A beads as described in Materials and methods. S-nitrosylated proteins were precipitated and the membranes were immunoblotted with anti-NF-κB p50 polyclonal antibody. The results demonstrate that p50 was S-nitrosylated. The findings are representative of two independent experiments. (**c**) Sensitization of PC-3 to TRAIL apoptosis by inhibition of NF-κB. PC-3 cells were treated with TRAIL (2.5 and 5.0 ng/ml) in the presence or absence of various concentrations of Bay11-7085 and apoptosis was assessed. The findings demonstrated that Bay11-7085 sensitizes PC-3 cells to TRAIL-mediated apoptosis. $*P<0.05$, $**P<0.02$, $***P<0.002$

Mechanism of DETANONOate-mediated sensitization to TRAIL apoptosis

We investigated the mechanism by which DETANONOate signals the cells leading to sensitization to TRAIL-mediated apoptosis. The effect of DETANONOate on the mitochondria was examined. DETANONOate significantly induced membrane depolarization of the mitochondria in PC-3 cells. In addition, TRAIL also significantly induced membrane depolarization, and the combination resulted in membrane depolarization that was equivalent to either DETANONOate or TRAIL used alone (Figure 5a). The effect of DETANONOate and TRAIL on the release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI) from the mitochondria was also examined. Both DETANONOate and TRAIL induced the release of both cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol, and the combination of DETANONOate and TRAIL resulted in more significant release of cytochrome *c*

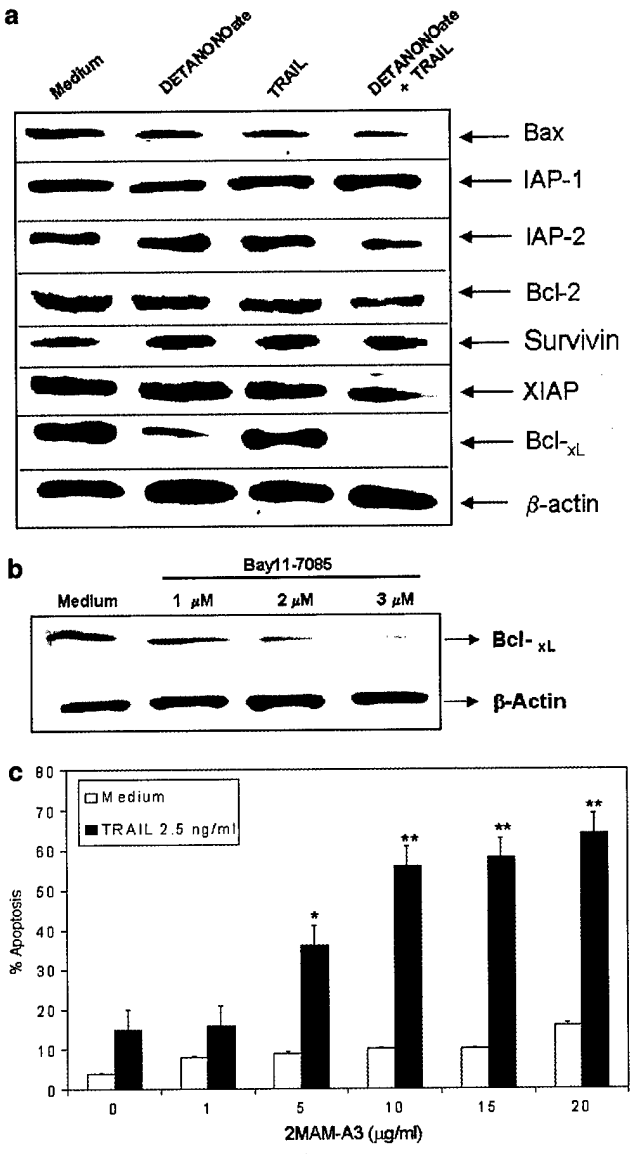

Figure 3 DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression and sensitization to TRAIL-mediated apoptosis. (**a**) PC-3 cells were grown in serum-free medium and the cells were treated or not treated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted and separated by SDS–PAGE and transferred onto nitrocellulose membranes as described in Materials and methods. DETANONOate selectively downregulated Bcl-$_{xL}$ expression. Treatment of PC-3 with different concentrations of the NF-κB inhibitor Bay11-7085 resulted in inhibition of Bcl-$_{xL}$ expression. (**b**) PC-3 cells were treated with different concentrations of the Bcl-$_{xL}$ inhibitor 2MAM-A3 for 5 h and then treated with TRAIL (2.5 ng/ml) for 18 h and analysed for apoptosis. The data show that 2MAM-A3 sensitizes PC-3 to TRAIL apoptosis. $*P=0.036$, $**P<0.02$

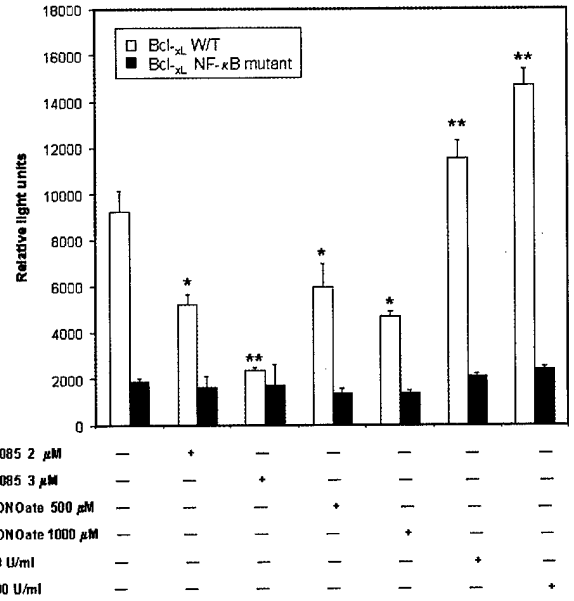

Figure 4 Inhibition of Bcl-xL transcription by DETANONOate. A Bcl-$_{xL}$ promoter fragment spanning −640 to −9 relative to the transcriptional start site (Bcl-$_{xL}$ WT promoter) and another fragment missing the NF-κB binding sequence (Bcl-$_{xL}$ ΔκB promoter) were cloned into the pGL2-Basic luciferase reporter vector (Lee *et al*., 1999). PC-3 cells were transfected with 20 μg of the indicated reporter plasmid and then treated with the specific NF-κB inhibitor Bay11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml). The samples were harvested 18 h after treatment and assessed for luciferase activity. The data show that DETANONOate inhibits Bcl-$_{xL}$ transcription by inhibition of luciferase activity. The data are representative of two experiments. $*P=0.031$, $**P<0.02$

and Smac/DIABLO (Figure 5b). In addition, there was little activation of procaspase 8 and procaspase 9 by either DETANONOate or TRAIL used alone, although the combination resulted in significant activation of procaspase 8 and procaspase 9 (Figure 5c). These findings demonstrate that DETANONOate selectively inhibits Bcl-$_{xL}$ expression (Figure 3a), and the activation of the mitochondria by both TRAIL and DETANONOate used in combination resulted in complementation and type II mitochondria-mediated sensitization of the cells to TRAIL-mediated apoptosis.

Discussion

This study presents evidence that the NO donor, DETANONOate, sensitizes androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis via inhibition of NF-κB activity and downregulation of Bcl-$_{xL}$ expression. The inactivation of NF-κB by DETANONOate was via S-nitrosylation of NF-κB p50. The role of NF-κB in the transcriptional activity of Bcl-$_{xL}$ expression was demonstrated by the use of NF-κB inhibitors and by the use of a luciferase reporter construct driving the Bcl-$_{xL}$ promoter. Treatment with DETANONOate or Bay11-7085 inhibited significantly luciferase activity whereas TNF-α augmented the basal activity. In contrast, removal of the putative NF-κB-binding sequence from the promoter resulted in low constitutive level of luciferase activity and this basal level was not affected by DETANONOate or by the NF-κB inhibitor. Inhibition of either NF-κB or Bcl-$_{xL}$ by chemical inhibitors sensitized significantly to TRAIL-mediated apoptosis. The synergy achieved in apoptosis by combination treatment was the

result of complementation in the activation of the type II mitochondrial pathway for apoptosis. Thus, both TRAIL and DETANONOate partially activate the mitochondria, with membrane potential depolarization and some release of cytochrome *c* and Smac/DIABLO, although each alone could not activate caspase 9. The combination of DETANONOate and TRAIL, however, resulted in caspase 9 and 3 activation and apoptosis. Altogether, these findings provide a novel mechanism of Bcl-$_{xL}$ regulation by NO via NF-κB inhibition and suggest that NO donors may be of potential therapeutic value as sensitizing agents when used in combination with TRAIL in the treatment of TRAIL-resistant tumor cells.

Our findings demonstrate that DETANONOate sensitized both androgen-dependent (LNCaP) and androgen-independent (DU145, PC-3, and CL-1) CaP cells to TRAIL-induced apoptosis and synergy was achieved. Previous findings from our laboratory have demonstrated that subtoxic concentrations of chemotherapeutic drugs like actinomycin D sensitized the above CaP tumor cells to TRAIL apoptosis (Zisman *et al*., 2001). Actinomycin D was shown to downregulate X-linked inhibitor of apoptosis (XIAP) selectively and, thus, facilitated the TRAIL-induced apoptotic pathway (Ng *et al*., 2002). The role of XIAP in resistance was corroborated in experiments showing that transfection with Smac/DIABLO, which inhibits inhibitor of apoptosis proteins (IAPs), sensitizes cells to TRAIL apoptosis in the absence of actinomycin D (Ng and Bonavida, 2002b). The present findings with DETANONOate, however, are different such that NO selectively inhibits NF-κB and Bcl-$_{xL}$ expression in the absence of modification of XIAP expression and sensitizes the cells to TRAIL apoptosis. These findings demonstrate that the regulation of apoptosis by TRAIL in the CaP cell lines studied may be influenced by various antiapoptotic members of the signaling pathway and the inhibition of one such member, such as XIAP or Bcl-$_{xL}$, was sufficient to reverse the resistance to TRAIL.

In CaP, NF-κB contributes to the progression to androgen independence and increases invasive and metastatic properties (Palayoor *et al*., 1999; Rayet and Gelinas, 1999). Basal levels of NF-κB are detected in normal prostatic epithelial cells and the androgen-dependent CaP cell line LNCaP (Palayoor *et al*., 1999; Huang *et al*., 2001). It has been reported that crosstalk occurs between NF-κB signaling and steroid receptor signaling pathways (Palvimo *et al*., 1996; McKay and Cidlowski, 2000). We show that treatment of LNCaP

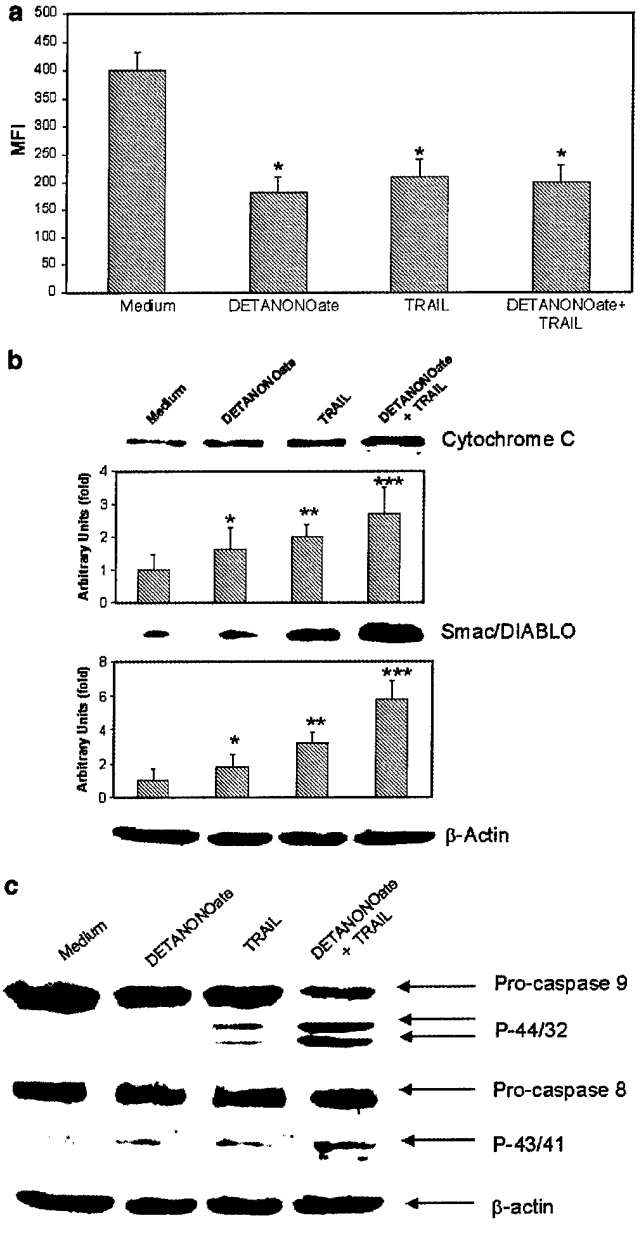

Figure 5 Mitochondrial membrane depolarization, release of cytochrome *c* and Smac/DIABLO into the cytosol, and activation of caspases 8 and 9. (**a**) Mitochondrial membrane activation. PC-3 cells were grown in FBS-free medium and treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. The PC-3 cells were then stained with DiOC6 and then analysed by flow cytometry. The findings demonstrate that DETANONOate, TRAIL, and the combination induce significant mitochondrial depolarization. The data represent the mean fluorescence intensity (MFI), and are the mean of three independent experiments. $*P<0.05$, medium *vs* cells treated. (**b**) Release of cytochrome *c* and Smac/DIABLO. PC-3 cells were grown in FBS-free medium and were treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted from the culture. The purified fraction of cytosolic protein was separated by SDS–PAGE and transferred onto the nitrocellulose membrane as described in Materials and methods. The membrane was stained with polyclonal anti-human cytochrome *c* antibody (top panel) or anti-Smac/DIABLO antibody (bottom panel). The blots represent one of two separate experiments. The data show that DETANONOate and TRAIL induce some release of both cytochrome and Smac/DIABLO, and the combination releases higher levels. The relative cytochrome *c* and Smac/DIABLO expression was determined by densitometric analysis of the blot. $*P<0.05$, $**P<0.03$, $***P<0.002$ medium *vs* cells treated. (**c**) Activation of caspases 8 and 9. PC-3 cells were treated as described above. The activation of caspases 8 and 9 was determined by Western blot. There was some activation of caspase 8 by DETANONOate and some activation of caspase 9 by TRAIL. However, the combination resulted in significant activation of both caspases

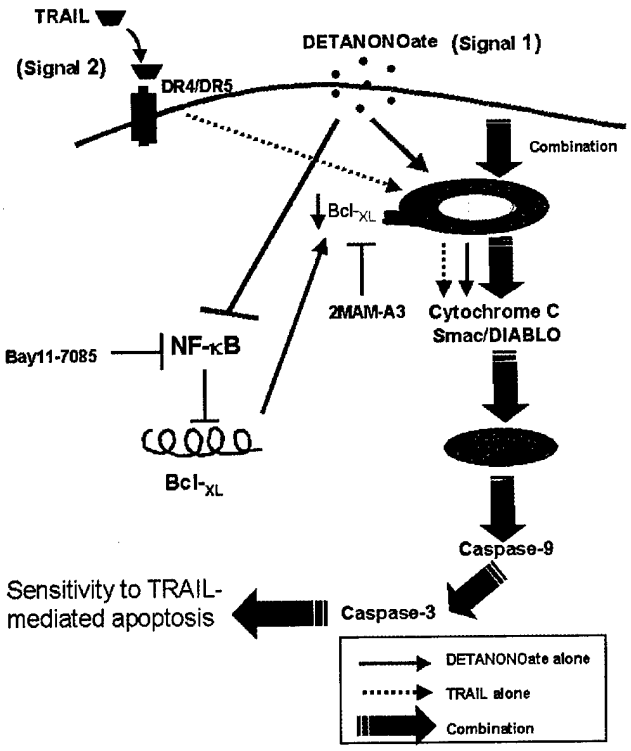

Figure 6 Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL. This figure schematically demonstrates that treatment of PC-3 cells with DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by DETANONOate, which partially inhibits NF-κB activity, and this leads to downregulation of Bcl-$_{xL}$ transcription. In addition, DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome *c* and Smac/DIABLO into the cytosol in the absence of downstream activation of caspase 9. Signal 2 is provided by TRAIL, which also partially activates the mitochondria with some release of cytochrome *c* and Smac/DIABLO in the absence of caspase 9 activation. However, the combination treatment results in significant activation of the mitochondria and release of high levels of cytochrome *c* and Smac/DIABLO, activation of caspases 9 and 3, resulting in apoptosis. The two-signal model is corroborated by the use of specific inhibitors in which inhibition of NF-κB by Bay11-7085 was sufficient to sensitize the CaP cells to TRAIL-induced apoptosis concomitant with downregulation of Bcl-$_{xL}$ expression. The role of Bcl-$_{xL}$ in the regulation of TRAIL apoptosis was corroborated by the use of the chemical inhibitor 2MAM-A3, which also sensitized the cells to apoptosis

with DHT sensitized the cells to TRAIL via inhibition of NF-κB. In contrast, androgen-independent CaP cells PC-3 and DU-145 have elevated NF-κB activity and this was confirmed here (data not shown). In addition, PC-3 and DU-145 cells have constitutively active IκB kinase complex (IKK), which activates NF-κB (Gasparian *et al*., 2002). Thus, constitutive activation of NF-κB plays a central role in the resistance to CaP cell line to therapeutic agents.

The present findings demonstrate that DETANONOate inhibits NF-κB activity. It has been shown that high levels of NO inhibit NF-κB activity by several mechanisms. For instance, DETANONOate inhibits the phosphorylation and subsequent degradation of IκB-α, which prevents nuclear localization of NF-κB (Katsuyama *et al*., 1998). Also, NO may quench reactive oxygen species that are responsible for the activation of NF-κB (Garban and Bonavida, 2001b). In addition, recent studies demonstrate that NO induces S-nitrosylation of NF-κB p50 and reduces its DNA-binding activity (Connely *et al*., 2001; Marshall and Stamler, 2001). NF-κB displays redox-sensitive DNA-binding activity (Chinenov *et al*., 1998; Tell *et al*., 1998). This redox sensitivity is conferred by a single cysteine residue within the DNA-binding site (Matthews *et al*., 1993; Marshall and Stamler, 2001). In this study, we demonstrate that NF-κB binding activity was significantly decreased after treatment with DETANONOate (Figure 2a). We also demonstrate that DETANONOate induced strongly S-nitrosylation of NF-κB p50 (Figure 2b) in agreement with the findings of Marshall and Stamler (2001) and Connely *et al*. (2001).

Recent studies demonstrated that Bcl-2 and Bcl-$_{xL}$ block apoptosis induced by physiological agents such as TRAIL in PC-3, DU-145, and LNCaP CaP cells (Rokhlin *et al*., 2001). In addition, overexpression of Bcl-$_{xL}$ in LNCaP and PC-3 cells desensitized the cells to the effects of cytotoxic chemotherapeutic agents (Li *et al*., 2001). However, downregulated endogenous levels of Bcl-$_{xL}$, but not Bcl-2, induced a marked increase in chemosensitivity (Lebedeva and Stain, 2000). These results suggest the important role of Bcl-$_{xL}$ in the resistance to apoptosis induced by cytotoxic agents like TRAIL in CaP. It is noteworthy that our results demonstrate that DETANONOate treatment induces selective downregulation of Bcl-$_{xL}$ expression and sensitizes the CaP cells to TRAIL-induced apoptosis. Further, inhibition of Bcl-$_{xL}$ function by 2MAM-A3 sensitizes the cells to TRAIL apoptosis. These findings corroborate the role of Bcl-$_{xL}$ in the regulation of resistance of CaP to chemotherapy and TRAIL.

The mechanism by which NO induces inhibition of Bcl-$_{xL}$ expression was examined. Previous findings demonstrated that the Bcl-$_{xL}$ promoter contains an element that binds NF-κB transcription factors and supports transcriptional activation by members of this family (Lee *et al*., 1999). It was possible that DETANONOate inhibits NF-κB and this, in turn, inhibits Bcl-$_{xL}$ transcription. We demonstrate here that DETANONOate inhibits Bcl-$_{xL}$ expression via inactivation of NF-κB activity. This was shown by using a luciferase reporter construct driving the Bcl-$_{xL}$ promoter. Treatment with DETANONOate or Bay 11-7085 (which selectively and irreversibly inhibits the induced phosphorylation of IκB without affecting the constitutive IκB-α phosphorylation; Pierce *et al*., 1997) significantly inhibited the high constitutive luciferase activity. However, there was little luciferase activity following the removal of the putative NF-κB-binding sequence from the promoter and neither DETANONOate nor Bay 11-7085 had any effect. These results directly demonstrate that Bcl-$_{xL}$ expression in PC-3 is primarily regulated by

NF-κB and inhibition of NF-κB, in turn, inhibits Bcl-$_{xL}$ transcription.

NO, synthesized from L-arginine by NO synthase, is a small, diffusible, highly reactive molecule with dual regulatory roles under physiological and pathological conditions (Schmidt and Walter, 1994). NO can promote apoptosis (proapoptosis) in some cells, whereas it inhibits apoptosis (antiapoptosis) in other cells. This dichotomy depends on the rate of NO production and the interaction with biological molecules such as iron, thiol, proteins, and reactive oxygen species (Schmidt, 1992; Stamler, 1994). High concentrations of NO and also long-lasting production of NO such as by DETANONOate used here act as proapoptotic modulators (Messmer and Brune, 1996; Poderoso *et al.*, 1996; Jun *et al.*, 1999; So *et al.*, 1998; Di Nardo *et al.*, 2000). The present findings are consistent with the proapoptotic effects of the high levels of NO used to sensitize CaP cells.

NO binds to cytochrome *c* oxidase (complex IV) in the mitochondrial electron transfer chain (Poderoso *et al.*, 1996). Under this condition, superoxide generated from mitochondria interacts with NO to form peroxynitrite, which induces mitochondrial dysfunction and cytochrome *c* release. NO also generates ceramide, which induces cytochrome *c* release from mitochondria (Ghafourifar *et al.*, 1999). Our results clearly show that DETANONOate induces activation of the mitochondria pathway, including mitochondrial membrane depolarization (Figure 3a) and some release of both cytochrome *c* and Smac/DIABLO (Figure 3b). The participation of the mitochondria is not complete because we demonstrate that downstream caspases are not activated. Caspase activation, however, resulted from the combination of DETNONOate and TRAIL. Recent studies have shown that caspase 8 activation is necessary but not sufficient for TRAIL-mediated apoptosis in prostate carcinoma cells (Rokhlin *et al.*, 2002), suggesting the important participation of the mitochondria-dependent pathway in TRAIL-mediated apoptosis. Further, our findings with DETANONOate are consistent with those of Lee *et al.* (2001), who reported that sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway.

This study demonstrates that the combination of NO donor and TRAIL can sensitize TRAIL-resistant CaP to TRAIL-induced apoptosis. This combination treatment is a result of two complementary signals induced by each agent alone (Ng and Bonavida, 2002a; schematically diagrammed in Figure 6). Signal 1 results from NO-induced perturbation of the mitochondria, inhibition of NF-κB activity, and downregulation of Bcl-$_{xL}$ expression. Signal 1 alone is not sufficient to promote the cells toward apoptosis. Signal 2 is induced by TRAIL, which activates the mitochondria slightly, but not sufficient to activate the apoptosome and induce apoptosis. However, combination of the two signals results in complementation and activation of the mitochondrial pathway and activation downstream of caspases 9 and 3 resulting in apoptosis. Thus, the findings of this report reveal that NO can selectively inhibit the expression of the antiapoptotic resistant factor Bcl-$_{xL}$ via inhibition of NF-κB activity. The findings also reveal new targets for intervention affecting NF-κB activity or Bcl-$_{xL}$ expression and whose modification may revert resistance of CaP to TRAIL apoptosis. Thus, NO donors or Bcl-$_{xL}$ inhibitors may be useful in the treatment of TRAIL-resistant tumors in combination with TRAIL or TRAIL agonists such as antibody against DR4/DR5 (DR: death receptor) (Ichikawa *et al.*, 2001).

Materials and methods

Reagents

The anti-Bcl-$_{xL}$ and anti-β-actin monoclonal antibodies were purchased from Santa Cruz (California, USA) and from Calbiochem (San Francisco, CA, USA), respectively. mAb anti-Bcl-2 was obtained from DAKO Corporation (Carpinteria, CA, USA). The polyclonal antibodies anti-XIAP, anti-IAP-1, anti-IAP-2, anticaspase 8, anticaspase 9, and survivin were obtained from Cell Signaling (San Diego, CA, USA), anti-cytochrome *c* from Pharmingen (San Diego, CA, USA), and anti-Smac/DIABLO from Alexis (San Diego, CA, USA). The human recombinant TRAIL and TNF-α were obtained from PeproTech Inc. (Rocky Hills, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-active caspase 3 and FITC-conjugated IgG were purchased from Pharmingen (San Diego, CA, USA). The NF-κB inhibitor Bay 11-7085 (specific inhibitor of IκBα phosphorylation; Pierce *et al.*, 1997) was obtained from Calbiochem (San Francisco, CA, USA), and the Bcl-$_{xL}$ inhibitor 2MAM-A3 (binds to the hydrophobic groove of Bcl-2 and Bcl-$_{xL}$) (Tzung *et al.*, 2001) was obtained from Biomol (Plymouth, PA, USA). The DETANONOate was obtained from Alexis (San Diego, CA, USA).

Cells and culture conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent LNCaP and the androgen-independent (Tso *et al.*, 2000) CL-1 (LNCaP-derived) cell lines were kindly provided by Dr Arie Belldegrun at UCLA. Cells were maintained as a monolayer in 80 mm^2 plates in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids. FBS (Life Technologies) was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/l R1881 methyltrienolone (New Life Science Products, Boston, MA, USA). The cell cultures were maintained as monolayers on plastic dishes and were incubated at 37°C and 5% carbon dioxide in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated FBS (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to treatments.

Cell treatments

Log-phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6×10^4 cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1, and PC-3 cells were synchronized by treatment with 1% FBS for 18 h prior to each experiment. The treatment of NCaP cells was in a medium with 1% of serum and the treatments of DU145, CL1, and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate, or the combination for 18 h. For the experiments of sensitization to TRAIL-mediated apoptosis by the NF-κB inhibitor Bay 11-7085, the cells were treated with different concentrations of Bay 11-7085 for 1 h and then treated with various concentrations of TRAIL for 18 h. For sensitization to TRAIL-mediated apoptosis by the Bcl-$_{xL}$ inhibitor 2MAM-A3, the cells were treated with different concentrations of 2MAM-A3 for 4 h, and then treated with TRAIL for 18 h.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice-cold 1 × phosphate-buffered saline (PBS) and were resuspended in 100 μl of the cytofix/cytoperm solution (Pharmingen, San Diego, CA, USA) for 20 min. Thereafter, the samples were washed twice with ice-cold 1 × perm/wash buffer solution (Pharmingen) and were stained with FITC-labeled anti-active caspase 3 mAb for 30 min (light protected). The samples were subsequently washed once with 1 × perm/wash buffer solution and 250 μl of 1 × PBS was added prior to flow cytometry analysis on a flow cytometer EPICS^R XL-MCL (Coulter, Co. Miami, FL, USA), with the System II™ Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50)

The S-nitrosylation of NF-κB p50 was analysed by immunoprecipitation assay. The cells were grown in the presence and absence of DETANONOate (0, 500, and 1000 μM) and then harvested and pelleted at 14 000 *g* for 2 min. The resulting cell pellets were resuspended and dissolved in 500 μl ice-cold components of radioimmunoprecipitation assay (RIPA) buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2 μg of rabbit anti-S-nitrosylated proteins polyclonal Ab (Calbiochem, San Diego, CA, USA) and were subsequently incubated with 30 μl Immuno-Pure Plus Immobilized protein A (Lindmark *et al*., 1983) (Pierce, Rockford, IL, USA) for 4 h at 4°C on a shaking platform. The lysates were centrifuged for 1 min at 14 000 *g* and the supernatants were discarded. The immunoprecipitates were washed twice with 1.0 ml of ice-cold RIPA buffer prior to assay. The immunoprecipitates were resolved on a 12% SDS–PAGE gel and subsequently immunoblotted with anti-NF-κB p50 polyclonal Ab (1 : 2000 dilution) (Active Motif, Carlsbad, CA, USA). The immunostaining was visualized by autoradiography.

Luciferase Bcl-$_{xL}$ promoter reporter assay

The Bcl-$_{xL}$ WT promoter luciferase (Bcl-x WT promoter) reporter plasmid and the Bcl-$_{xL}$ promoter missing the NF-κB-binding sequence (Bcl-x κB promoter) have been previously characterized (Lee *et al*., 1999). PC-3 cells were transfected by electroporation using pulses at 250 V/975 μF (Bio-Rad), with 20 μg of Bcl-x WT promoter or Bcl-x κB promoter. After transfection, the cells were allowed to recover overnight and were cultured in six-well plates. Cells were treated with the specific NF-κB inhibitor Bay 11-7085 (2 or 3 μM), NO donor DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml) for 18 h. Cells were then harvested in 1 × lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate.

Measurement of mitochondrial membrane depolarization

The mitochondria-specific dye 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (Molecular Probes Inc., Eugene, OR, USA) was used to measure the mitochondrial potential. PC-3 cells were grown in six-well plates and were treated with TRAIL (2.5 ng/ml) and/or DETANONOate (1000 μM) simultaneously. After treatments, the cells were collected at 18 h. A total of 50 μl of 40 μM ($DiOC_6$) was loaded to stain the cells for 30 min immediately after the cells were collected. The cells were detached by using PBS supplemented with 0.5 μM ethylenediaminetetraacetic acid (EDTA), washed twice in PBS, resuspended in 1 ml of PBS, and analysed by flow cytometry as reported (Ng *et al*., 2002).

Western blot analysis

PC-3 cells were cultured at a low FBS concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with TRAIL (2.5 ng/ml), DETANONOate (1000 μM), or the combination. The cells were then lysed at 4°C in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN, USA). Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). An aliquot of total protein lysate was diluted in an equal volume of 2 × SDS sample buffer, 6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 10 min. The cell lysates (40 μg) were then electrophoresed on 12% SDS–PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi *et al*., 2001). Levels of β-actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) using the public domain NIH Image J Program (also available via the internet).

Isolation of cytosolic fraction and determination of cytochrome c *and Smac/DIABLO content*

PC-3 cells were grown under the conditions explained for Western blot. At the end of the incubation period, the cells were recovered with 1 × PBS/EDTA, washed with 1.0 × PBS/0.1% BSA and resuspended in two volumes of homogenization buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM 1,4-dithiothreitol (DTT), one tablet of Complete Mini protease inhibitor cocktail in 250 mM sucrose medium). After 30 min on ice, the cells were disrupted by 40 strokes of a dounce glass homogenizer using a loose pestle (Bellco Glass Inc., Vineland, NJ, USA). The homogenate was centrifuged at 2500 *g* at 4°C

for 5 min to remove nuclei and unbroken cells. The mitochondria were pelleted by spinning the homogenate at 16 000 *g* at 4°C for 30 min. The supernatant was removed and filtered through 0.1 μm Ultrafree MC filters (Millipore) to obtain the cytosolic fraction and was spun down at 16 000 *g* at 4°C for 15 min. The protein concentration of the supernatant was determined by the DC assay kit and was mixed with 2× Laemmli sample buffer and analysed by SDS–PAGE for determination of cytochrome *c* and Smac/DIABLO contents in the cytosolic fraction as previously reported (Jazirehi *et al.*, 2003).

Nuclear extracts preparation

Nuclear extract preparations were carried out as previously described by our laboratory (Garban and Bonavida, 2001b). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA, USA). After washing, cells were lysed in 1 ml of NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) on ice for 5 min. Samples were centrifuged at 300 *g* at 4°C for 5 min. The pellet was washed twice in NP-40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN, USA). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at −80°C.

EMSA

Nuclear proteins (5 μg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB using EMSA Kit Panomics™ (Panomics Inc., Redwood City, CA, USA) following the manufacturer's instructions (Vega *et al.*, 2004). A measure of 10 μl was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide sequences for NF-κB are as follows: 5′-AGTTGAGGGGACTT TCCCAGGC-3′ (Harada *et al.*, 1994). Relative concentrations were assessed by densitometric analysis as mentioned above.

Isobologram analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/DETONONOate combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5, and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 μM) as described (Berenbaum, 1978). Combinations yielding a cytotoxicity of 30±5% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose–response curves using active caspase 3 analysis for LNCaP, DU145, CL-1, and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18 h.

Statistical analysis

The experimental values were expressed as the mean±s.d. for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's *t*-test was used for comparison between two groups. Significant differences were considered for probabilities <5% ($P<0.05$).

Abbreviations

Bcl-$_{xL}$, Bcl-2 related gene; CaP, prostate cancer; DETANONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate; DHT, 5-α dihydrotestosterone; DR, death receptor; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IAP, inhibitor of apoptosis protein; IKK, IκB kinase complex; JNK, c-Jun N-terminal kinase; 2MAM-A3, 2-methoxyantimycin A_3; NF-κB, nuclear factor κB; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; RIPA, radioimmunoprecipitation assay (buffer); SDS, sodium dodecyl sulfate; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbolacetate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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Back

Abstract Number: 4826

Regulation of prostate cancer sensitivity to apoptosis by CDDP by downregulation of XIAP and Bcl-$_{xL}$ expression via inhibition of constitutive NF-κB activity

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Prostate cancer patients develop chemoresistance following initial treatment. Several studies have been addressed to determine the underlying mechanisms of resistance and included the development of the MDR phenotype, dysregulation of the apoptotic signaling pathways, etc. We and others have demonstrated in CaP cell lines that NF-κB is constitutively activated and regulates survival and growth of the tumor cells. We have demonstrated that PC-3 and DU-145 synthesize and secrete cytokines such as TNF-α and IL-6 that regulate NF-κB activity via an autocrine paracrine pathway. Further, these cytokines were found to regulate drug resistance. Therefore, we hypothesized that NF-κB may regulate drugs-induced apoptosis via dysregulation of the apoptotic signaling pathways and hence inhibition of NF-κB may chemosensitize the cells. The objective of this study was to test this hypothesis and delineate the gene products that are transcriptionally regulated by NF-κB and contribute to chemoresistance. We demonstrate that treatment of PC-3 cells with NF-κB inhibitors sensitized the cells to CDDP-induced apoptosis and synergy was achieved. Further, inhibition of NF-κB by a nitric oxide donor (DETANONOate), via the S-nitrosylation of p50, also resulted in chemosenstization. The effect of NF-κB on the apoptosis signaling pathways, as determined by western, revealed that there was selective downregulation of the anti-apoptotic XIAP and Bcl-$_{xL}$ gene products following inhibition of NF-κB. The role of these proteins in chemosensitization was corroborated by the use of specific inhibitors. These findings suggest that tumor cells develop a self-contained mechanism to resist drug-induced apoptosis by tumor-derived autocrine/paracrine factors which activate NF-κB and secure cell growth and survival. Further, the present findings suggest that NF-κB, XIAP and Bcl-$_{xL}$ are targets for intervention in the reversal of drug resistance. Supported by a grant from the Department of Defense (US Army DAMD 17-02-1-0023), Jonsson Comprehensive Cancer Center (MV, AJ), UCLA SPORE in Prostate Cancer (P50 CA92131-01A1), Fogarty (SH-Y, MV, HG), and UC MEXUS (SH-Y).

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Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-κB and inhibition of Bcl-$_{xL}$ expression

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues and this prompted its potential therapeutic application in cancer. However, not all cancers are sensitive to TRAIL-mediated apoptosis and, therefore, TRAIL-resistant cancer cells must be sensitized first to become sensitive to TRAIL. Treatment of prostate cancer (CaP) cell lines (DU145, PC-3, CL-1, and LNCaP) with nitric oxide donors (e.g. (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate (DETANONOate)) sensitized CaP cells to TRAIL-induced apoptosis and synergy was achieved. The mechanism by which DETANONOate mediated the sensitization was examined. DETANONOate inhibited the constitutive NF-κB activity as assessed by EMSA. Also, p50 was S-nitrosylated by DETANONOate resulting in inhibition of NF-κB. Inhibition of NF-κB activity by the chemical inhibitor Bay 11-7085, like DETANONOate, sensitized CaP to TRAIL apoptosis. In addition, DETANONOate downregulated the expression of Bcl-2 related gene (Bcl-$_{xL}$) which is under the transcriptional regulation of NF-κB. The regulation of NF-κB and Bcl-$_{xL}$ by DETANONOate was corroborated by the use of Bcl-$_{xL}$ and Bcl-x κB reporter systems. DETANONOate inhibited luciferase activity in the wild type and had no effect on the mutant cells. Inhibition of NF-κB resulted in downregulation of Bcl-$_{xL}$ expression and sensitized CaP to TRAIL-induced apoptosis. The role of Bcl-$_{xL}$ in the regulation of TRAIL apoptosis was corroborated by inhibiting Bcl-$_{xL}$ function by the chemical inhibitor 2-methoxyantimycin A_3 and this resulted in sensitization of the cells to TRAIL apoptosis. Signaling by DETANONOate and TRAIL for apoptosis was examined. DETANONOate altered the mitochondria by inducing membrane depolarization and releasing modest amounts of cytochrome *c* and Smac/DIABLO in the absence of downstream activation of caspases 9 and 3. However, the combination of DETANONOate and TRAIL resulted in activation of the mitochondrial pathway and activation of caspases 9 and 3, and induction of apoptosis. These findings demonstrate that DETANONOate-mediated sensitization of CaP to TRAIL-induced apoptosis is via inhibition of constitutive NF-κB activity and Bcl-$_{xL}$ expression.

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Keywords: NF-κB; prostate cancer; nitric oxide; TRAIL; apoptosis

Introduction

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutics such as drugs, irradiation, and immunotherapy since most of their primary cytotoxic effects are through apoptosis (Ng and Bonavida, 2002a; Hersey and Zhang, 2003). Therefore, after the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, antitumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (Ashkenazi and Dixit, 1999; Ashkenazi *et al.*, 1999). TRAIL has been considered a new therapeutic, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (Ashkenazi *et al.*, 1999; De Jong *et al.*, 2001; Wajant *et al.*, 2002; Chawla-Sarkar *et al.*, 2003). However, many tumor cells have been shown to be resistant to TRAIL (Zisman *et al.*, 2001; Ng *et al.*, 2002; Bouralexis *et al.*, 2003; Tillman *et al.*, 2003). We and others have reported that various sensitizing agents like chemotherapeutic drugs (Zisman *et al.*, 2001; Munshi *et al.*, 2002), cytokines (Park *et al.*, 2002), and inhibitors (Nyormoi *et al.*, 2003) are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

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Prostate cancer (CaP) cells have been shown to exhibit constitutive nuclear factor κB (NF-κB) activity (Suh *et al.*, 2002). It has been recently reported that NF-κB can regulate the sensitivity of target cells to TRAIL apoptosis in hepatoma cells (Shigero *et al.*, 2003). In addition, it has been reported that CaP cells overexpress Bcl-2 related gene (Bcl-$_{xL}$), which negatively regulates tumor cells sensitivity to drug-mediated apoptosis (Raffo *et al.*, 1995). Studies on Bcl-$_{xL}$ gene transcription demonstrate that Bcl-$_{xL}$ is regulated in part by NF-κB (Mori *et al.*, 2001). Thus, constitutive expression of NF-κB in CaP may regulate the constitutive expression of Bcl-$_{xL}$. We have reported that nitric oxide (NO) donors can sensitize tumor cells to FasL and tumor necrosis factor alpha (TNF-α)-mediated apoptosis (Garban and Bonavida, 2001a, b). Further, we (Huerta-Yepez *et al.*, 2003) and others (Lee *et al.*, 2001; Secchiero *et al.*, 2001) reported that (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1, 2-diolate (DETANONOate) can also sensitize tumor cells to TRAIL-mediated apoptosis.

The mechanism underlying the NO-mediated sensitization to TRAIL is not known. We hypothesized that NO-mediated sensitization of CaP cells to apoptosis may be due to NO-induced inhibition of constitutive NF-κB activity and this, in turn, will result in the downregulation of Bcl-$_{xL}$ transcription and expression. Hence, downregulation of the antiapoptotic gene product Bcl-$_{xL}$ will result in the sensitization of CaP cells to TRAIL-mediated apoptosis. This study was designed to test this hypothesis and the followings were investigated: (1) Does NO sensitize androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis? (2) Does NO inhibit constitutive NF-κB activity resulting in inhibition of Bcl-$_{xL}$ expression? (3) Do inhibitors of NF-κB and Bcl-$_{xL}$ mimic NO and sensitize CaP to TRAIL-mediated apoptosis? And (4) by what mechanism does NO modify the apoptotic signaling pathway and sensitize CaP to TRAIL-mediated apoptosis?

Results

Sensitization of CaP cell lines to TRAIL-mediated apoptosis by DETANONOate

Our previous findings have demonstrated that CaP cell lines (LNCaP, DU-145, PC-3, and CL-1) are relatively resistant to TRAIL-mediated apoptosis (Zisman *et al.*, 2001; Ng *et al.*, 2002), and are shown in Figure 1a. However, pretreatment of CaP cell lines with the NO donor DETANONOate resulted in significant potentiation of apoptosis by TRAIL for the four cell lines tested. The extent of potentiation was a function of the concentration of TRAIL used (Figure 1a). The sensitization by DETANONOate was synergistic as determined by isobologram analysis (Figure 1b). We selected PC-3 as a model system for further investigation. Treatment of PC-3 cells with various concentrations of DETANONOate sensitized the cells to TRAIL-induced apoptosis, and the extent of apoptosis was a function of the concentration of DETANONOate used (Figure 1c). In addition to apoptosis, NO, TRAIL, and the combination inhibited cell proliferation significantly (Figure 1d). These findings demonstrate that DETANONOate sensitizes androgen-dependent and -independent CaP tumor cell lines to TRAIL-mediated apoptosis and synergy is achieved. Previous findings demonstrated that the androgen 5-α dihydrotestosterone (DHT) sensitizes LNCaP to 12-*O*-tetradecanoylphorbolacetate (TPA)-induced apoptosis (Altuwaijri *et al.*, 2003). We examined whether DHT also sensitizes LNCaP to TRAIL apoptosis. We observed that treatment of LNCaP with DHT sensitizes the cells to TRAIL (Table 1).

DETANONOate inhibits NF-κB activity and inhibition of NF-κB sensitizes PC-3 to TRAIL apoptosis

We examined the effect of DETANONOate on NF-κB activity in PC-3 cells. The cells were treated with DETANONOate (500 and 1000 μM) and tested for NF-κB activity by EMSA. In addition, we used the NF-κB inhibitor, Bay 11-7085, at different concentrations as control for inhibition of NF-κB activity. Figure 2a demonstrates that DETANONOate inhibits NF-κB activity significantly and the inhibition at 1000 μM was much higher than the inhibition at 500 μM. As expected, the Bay 11-7085 inhibitor also significantly inhibited NF-κB activity, and the inhibition was a function of the concentration of Bay 11-7085 used (Figure 2a).

It has been reported that the DNA-binding activity of NF-κB p50 can be modified by NO and p50 becomes S-nitrosylated and inhibits NF-κB activity (Matthews *et al.*, 1996; Dela Torre *et al.*, 1997; Marshall and Stamler, 2001). Thus, we examined whether DETANONOate treatment of PC-3 cells induces S-nitrosylation of p50. PC-3 cells were grown in the absence or presence of DETANONOate (500 or 1000 μM) for 18 h and total cell lysates were prepared and immunoprecipitation assay was performed as described in Materials and methods. Using anti-S-nitrosylated antibody, the S-nitrosylated proteins were immunoprecipitated and were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with

Table 1 DHT sensitizes LNCaP to TRAIL-mediated apoptosis

	TRAIL (ng/ml)		
DHT (nM)	*0*	*5*	*10*
0	5.1±1	12±2.1	17±3.8
10	6.6±0.9	18±1.1*	24±5.1*
20	6.9±1.1	23±6.1*	30.6±6.3**

LNCaP cells were treated or left untreated with DHT (10 or 20 nM) for 24 h and then treated with recombinant TRAIL (5 or 10 ng/ml) for 18 h. The cells were harvested and apoptosis was determined for cells with active caspase 3 staining by flow. The data show that DHT sensitizes LNCaP to TRAIL-mediated apoptosis. The data represent the mean of two independent experiments. *$P<0.04$, **$P<0.02$ compared with the cells treated with DHT alone

anti-NF-κB p50 antibody. S-nitrosylation of p50 was significantly enhanced following DETANONOate treatment (Figure 2b).

The relationship between DETANONOate-mediated inhibition of NF-κB and sensitization to TRAIL was examined. PC-3 cells were treated with various concentrations of Bay 11-7085 (1–5 μM) and TRAIL (5 and 10 ng/ml). Treatment with Bay 11-7085 significantly potentiated the sensitivity of PC-3 to TRAIL-mediated apoptosis, and the degree of apoptosis was a function of the concentration used (Figure 2c).

These findings demonstrate that DETANONOate inhibits NF-κB activity and results in the sensitization of PC-3 to TRAIL-induced apoptosis. Further, the results suggest that DETANONOate-mediated sensitization is via inactivation of NF-κB.

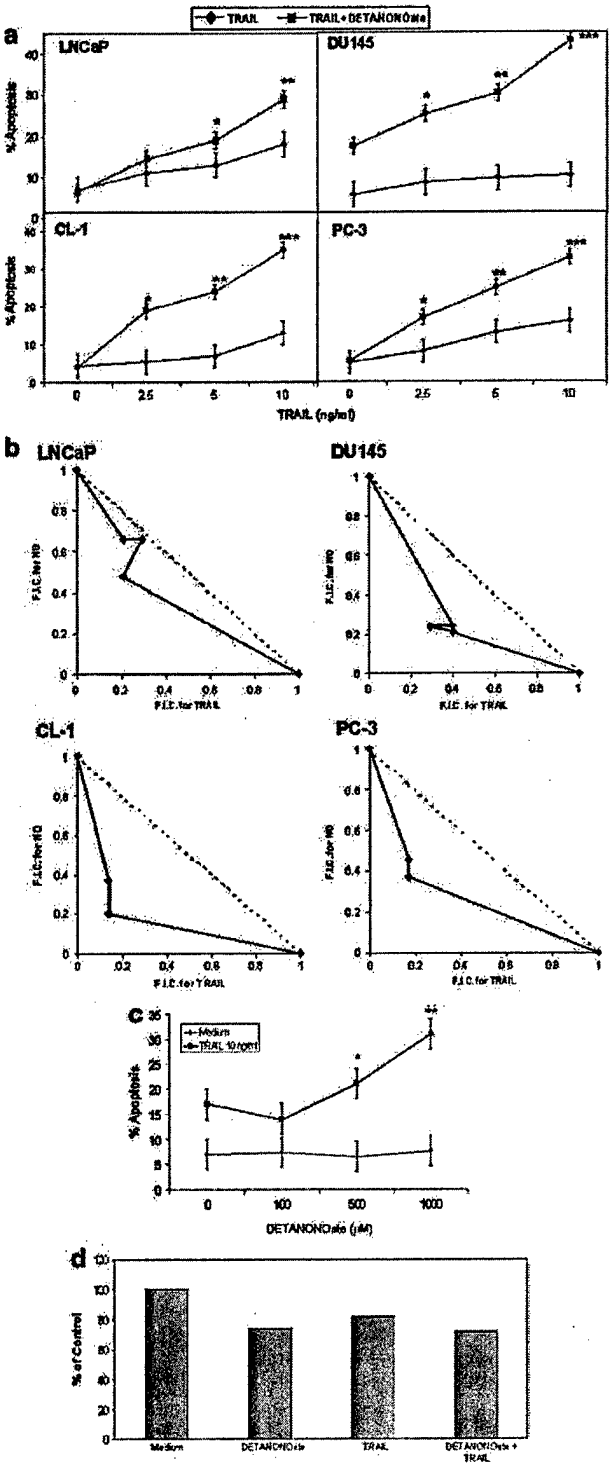

Figure 1 DETANONOate sensitizes CaP cell lines to TRAIL-mediated apoptosis. **(a)** The CaP cell lines DU145, CL-1, and PC-3 were grown in FBS-free medium and LNCaP cells were grown in a medium with 1% FBS. The cell lines were treated with different concentrations of TRAIL (0, 2.5, and 5 ng/ml) in the presence or absence of DETANONOate (1000 μM) for 18 h at 37° in a 5% CO_2 incubator. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analysed by flow cytometry as described in Materials and methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. $*P<0.05$, $**P<0.02$, $***P<0.004$. **(b)** This figure establishes synergy as determined by isobologram analysis. **(c)** PC-3 cells were grown in FBS-free medium and were treated with TRAIL (5 ng/ml) in the presence or absence of different concentrations of DETANONOate (100, 500, and 1000 μM) for 18 h and analysed for apoptosis. Significant sensitization was observed at DETANONOate concentrations of 500 and 1000 μM. **(d)** The PC-3 cells were treated with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), and the combination, and viable cell recovery was examined microscopically by Trypan blue dye exclusion at 24 h. The data show that all agents inhibited cell proliferation

DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression and sensitization to TRAIL

DETANONOate selectively inhibited Bcl-$_{xL}$ expression in PC-3 with little effect on other pro- and antiapoptotic gene products examined (Figure 3a). TRAIL has no effect on any of the gene products examined. It has been reported that Bcl-$_{xL}$ transcription is regulated in part by NF-κB (Mori *et al.*, 2001; Sevilla *et al.*, 2001). Thus, it was possible that DETANONOate-mediated inhibition of NF-κB (Figure 2a) was responsible for the observed DETANONOate-mediated inhibition of Bcl-$_{xL}$ expression (Figure 3a). This was confirmed by demonstrating that treatment of PC-3 with the NF-κB inhibitor Bay 11-7085, like DETANONOate, also inhibited Bcl-$_{xL}$ expression (Figure 3b). Therefore, it was possible that sensitization of PC-3 by DETANONOate to TRAIL-induced apoptosis was due in part to downregulation of Bcl-$_{xL}$ expression via inhibition of NF-κB. Accordingly, inhibition of Bcl-$_{xL}$ expression should sensitize PC-3, like NO, to TRAIL-induced apoptosis. Treatment of PC-3 with the Bcl-$_{xL}$ inhibitor 2-methoxyantimycin A_3 (2MAM-A3) (Tzung *et al.*, 2001) resulted in significant sensitization of the cells to TRAIL-induced apoptosis. The potentiation was a function of the concentration of 2MAM-A3 used (Figure 3c). These findings suggest that Bcl-$_{xL}$ is the dominant resistant factor in PC-3 cells for TRAIL-induced apoptosis, and Bcl-$_{xL}$ inhibition by DETANONOate via NF-κB

inactivation may be responsible for sensitization to TRAIL.

It has been reported that NF-κB activity plays an important role in the transcriptional regulation of Bcl-$_{xL}$ (Mori *et al.*, 2001; Sevilla *et al.*, 2001). To determine whether NF-κB activity is required for Bcl-$_{xL}$ transcription and to determine how DETANONOate induces selective inhibition of Bcl-$_{xL}$ via NF-κB, transient transfection assays were performed. PC-3 cells were transfected with the Bcl-x WT promoter and Bcl-x κB promoter reporter plasmids. At 24 h after transfection, the cells were treated with either Bay 11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or optimal concentrations of TNF-α (50 or 100 U/ml) for 18 h. Both DETANONOate treatment and Bay 11-7085 treatment induced significant inhibition of Bcl-$_{xL}$ transcription, and the extent of inhibition was concentration dependent. In contrast, activation of NF-κB by TNF-α treatment induced a significant increase in Bcl-$_{xL}$ transcription (Figure 4). The basal luciferase activity was significantly reduced in the mutant (5×) compared to wild type, suggesting that Bcl-$_{xL}$ transcription in PC-3 is primarily regulated by NF-κB. In contrast to the findings in the wild type, the different treatments did not affect the cells transfected with the Bcl-x κB promoter (Figure 4). These results indicate that Bcl-$_{xL}$ transcription in PC-3 is in large part regulated by NF-κB, and inhibition of NF-κB by DETANONOate is responsible for DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression.

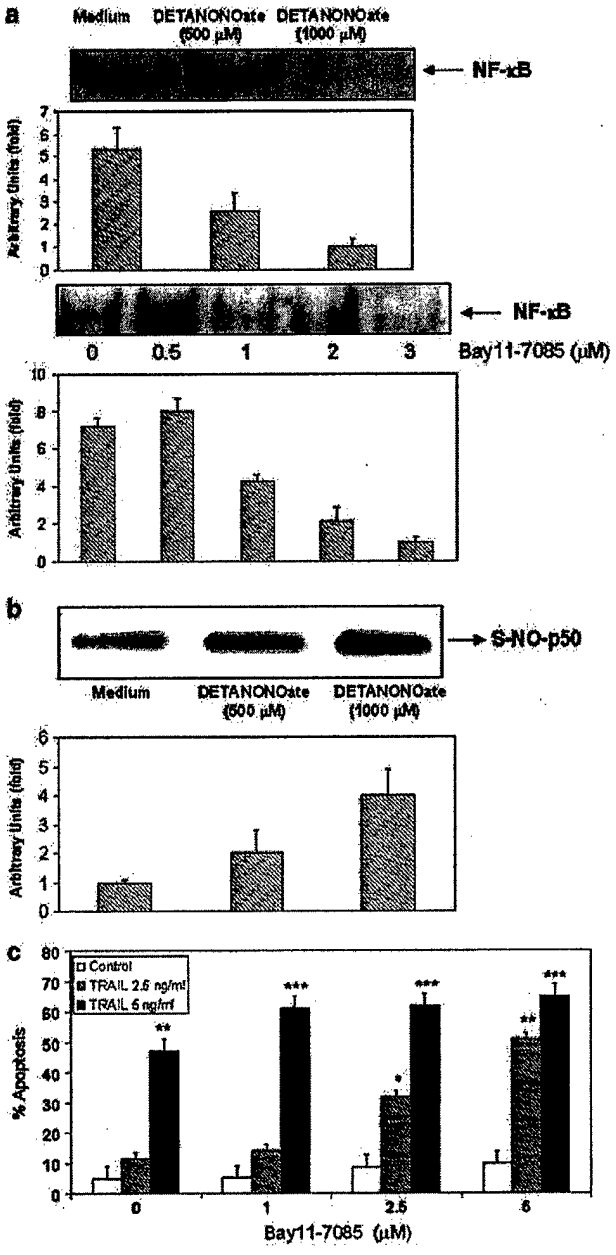

Figure 2 NF-κB is involved in TRAIL-mediated apoptosis in PC-3 cells. (**a**) Inhibition of NF-κB activity. Nuclear extracts from PC-3 cells grown in FBS-free medium were treated or left untreated with DETANONOate (500 or 1000 μM) (top panel), or treated with different concentrations of the specific NF-κB inhibitor Bay 11-7085 (0, 0.5, 1, 2, and 3 μM) (bottom panel), and were analysed by EMSA to assess NF-κB DNA-binding activity. Relative NF-κB binding activity was determined by densitometry analysis. The findings demonstrate that treatment of PC-3 cells with DETANONOate results in inhibition of NF-κB activity. (**b**) Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50) upon DETANONOate (500 and 1000 μM, 18 h) treatment. Total cell lysates were used in an immunoprecipitation assay using protein A beads as described in Materials and methods. S-nitrosylated proteins were precipitated and the membranes were immunoblotted with anti-NF-κB p50 polyclonal antibody. The results demonstrate that p50 was S-nitrosylated. The findings are representative of two independent experiments. (**c**) Sensitization of PC-3 to TRAIL apoptosis by inhibition of NF-κB. PC-3 cells were treated with TRAIL (2.5 and 5.0 ng/ml) in the presence or absence of various concentrations of Bay11-7085 and apoptosis was assessed. The findings demonstrated that Bay11-7085 sensitizes PC-3 cells to TRAIL-mediated apoptosis. $^{*}P<0.05$, $^{**}P<0.02$, $^{***}P<0.002$

Mechanism of DETANONOate-mediated sensitization to TRAIL apoptosis

We investigated the mechanism by which DETANONOate signals the cells leading to sensitization to TRAIL-mediated apoptosis. The effect of DETANONOate on the mitochondria was examined. DETANONOate significantly induced membrane depolarization of the mitochondria in PC-3 cells. In addition, TRAIL also significantly induced membrane depolarization, and the combination resulted in membrane depolarization that was equivalent to either DETANONOate or TRAIL used alone (Figure 5a). The effect of DETANONOate and TRAIL on the release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI) from the mitochondria was also examined. Both DETANONOate and TRAIL induced the release of both cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol, and the combination of DETANONOate and TRAIL resulted in more significant release of cytochrome *c*

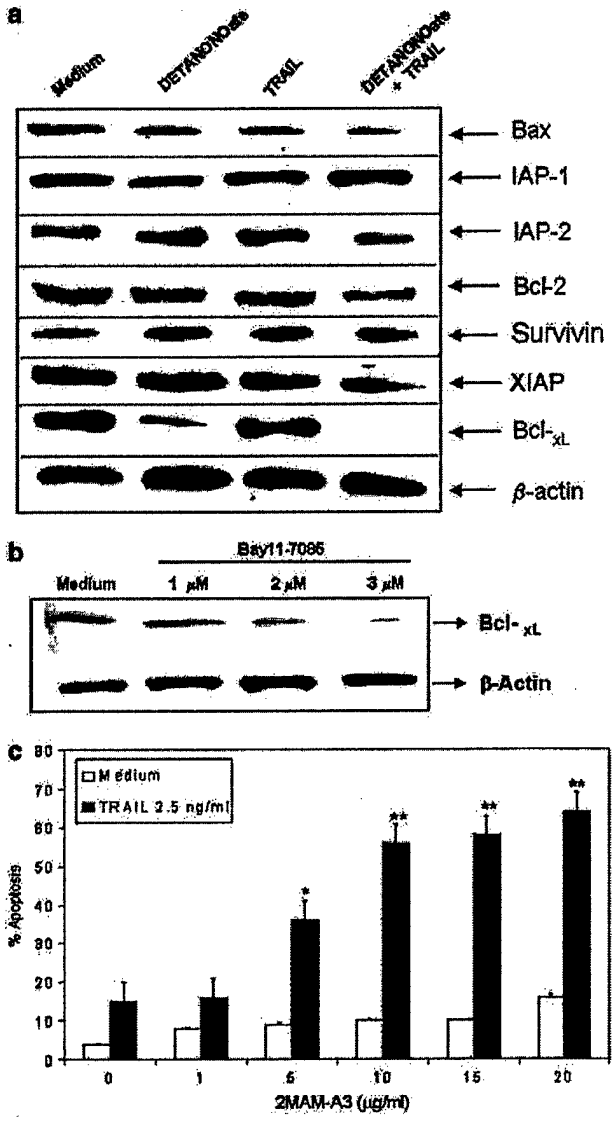

Figure 3 DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression and sensitization to TRAIL-mediated apoptosis. (**a**) PC-3 cells were grown in serum-free medium and the cells were treated or not treated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted and separated by SDS–PAGE and transferred onto nitrocellulose membranes as described in Materials and methods. DETANONOate selectively downregulated Bcl-$_{xL}$ expression. Treatment of PC-3 with different concentrations of the NF-κB inhibitor Bay11-7085 resulted in inhibition of Bcl-$_{xL}$ expression. (**b**) PC-3 cells were treated with different concentrations of the Bcl-$_{xL}$ inhibitor 2MAM-A3 for 5 h and then treated with TRAIL (2.5 ng/ml) for 18 h and analysed for apoptosis. The data show that 2MAM-A3 sensitizes PC-3 to TRAIL apoptosis. $^{*}P=0.036$, $^{**}P<0.02$

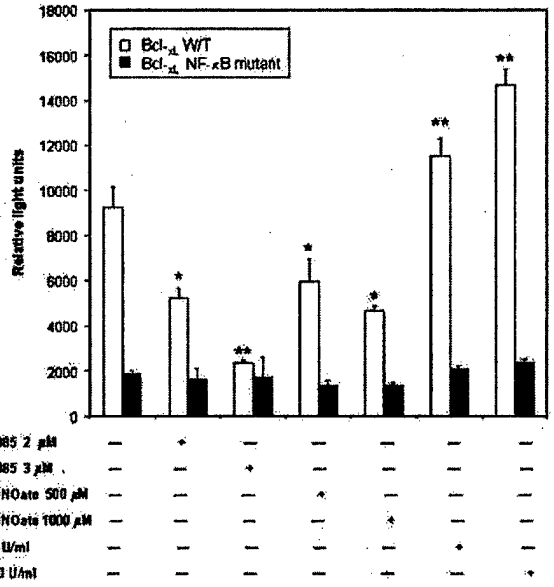

Figure 4 Inhibition of Bcl-xL transcription by DETANONOate. A Bcl-$_{xL}$ promoter fragment spanning −640 to −9 relative to the transcriptional start site (Bcl-$_{xL}$ WT promoter) and another fragment missing the NF-κB binding sequence (Bcl-$_{xL}$ ΔκB promoter) were cloned into the pGL2-Basic luciferase reporter vector (Lee *et al*., 1999). PC-3 cells were transfected with 20 μg of the indicated reporter plasmid and then treated with the specific NF-κB inhibitor Bay11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml). The samples were harvested 18 h after treatment and assessed for luciferase activity. The data show that DETANONOate inhibits Bcl-$_{xL}$ transcription by inhibition of luciferase activity. The data are representative of two experiments. $^{*}P=0.031$, $^{**}P<0.02$

and Smac/DIABLO (Figure 5b). In addition, there was little activation of procaspase 8 and procaspase 9 by either DETANONOate or TRAIL used alone, although the combination resulted in significant activation of procaspase 8 and procaspase 9 (Figure 5c). These findings demonstrate that DETANONOate selectively inhibits Bcl-$_{xL}$ expression (Figure 3a), and the activation of the mitochondria by both TRAIL and DETANONOate used in combination resulted in complementation and type II mitochondria-mediated sensitization of the cells to TRAIL-mediated apoptosis.

Discussion

This study presents evidence that the NO donor, DETANONOate, sensitizes androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis via inhibition of NF-κB activity and downregulation of Bcl-$_{xL}$ expression. The inactivation of NF-κB by DETANONOate was via S-nitrosylation of NF-κB p50. The role of NF-κB in the transcriptional activity of Bcl-$_{xL}$ expression was demonstrated by the use of NF-κB inhibitors and by the use of a luciferase reporter construct driving the Bcl-$_{xL}$ promoter. Treatment with DETANONOate or Bay11-7085 inhibited significantly luciferase activity whereas TNF-α augmented the basal activity. In contrast, removal of the putative NF-κB-binding sequence from the promoter resulted in low constitutive level of luciferase activity and this basal level was not affected by DETANONOate or by the NF-κB inhibitor. Inhibition of either NF-κB or Bcl-$_{xL}$ by chemical inhibitors sensitized significantly to TRAIL-mediated apoptosis. The synergy achieved in apoptosis by combination treatment was the

result of complementation in the activation of the type II mitochondrial pathway for apoptosis. Thus, both TRAIL and DETANONOate partially activate the mitochondria, with membrane potential depolarization and some release of cytochrome *c* and Smac/DIABLO, although each alone could not activate caspase 9. The combination of DETANONOate and TRAIL, however, resulted in caspase 9 and 3 activation and apoptosis. Altogether, these findings provide a novel mechanism of Bcl-$_{xL}$ regulation by NO via NF-κB inhibition and suggest that NO donors may be of potential therapeutic value as sensitizing agents when used in combination with TRAIL in the treatment of TRAIL-resistant tumor cells.

Our findings demonstrate that DETANONOate sensitized both androgen-dependent (LNCaP) and androgen-independent (DU145, PC-3, and CL-1) CaP cells to TRAIL-induced apoptosis and synergy was achieved. Previous findings from our laboratory have demonstrated that subtoxic concentrations of chemotherapeutic drugs like actinomycin D sensitized the above CaP tumor cells to TRAIL apoptosis (Zisman *et al*., 2001). Actinomycin D was shown to downregulate X-linked inhibitor of apoptosis (XIAP) selectively and, thus, facilitated the TRAIL-induced apoptotic pathway (Ng *et al*., 2002). The role of XIAP in resistance was corroborated in experiments showing that transfection with Smac/DIABLO, which inhibits inhibitor of apoptosis proteins (IAPs), sensitizes cells to TRAIL apoptosis in the absence of actinomycin D (Ng and Bonavida, 2002b). The present findings with DETANONOate, however, are different such that NO selectively inhibits NF-κB and Bcl-$_{xL}$ expression in the absence of modification of XIAP expression and sensitizes the cells to TRAIL apoptosis. These findings demonstrate that the regulation of apoptosis by TRAIL in the CaP cell lines studied may be influenced by various antiapoptotic members of the signaling pathway and the inhibition of one such member, such as XIAP or Bcl-$_{xL}$, was sufficient to reverse the resistance to TRAIL.

In CaP, NF-κB contributes to the progression to androgen independence and increases invasive and metastatic properties (Palayoor *et al*., 1999; Rayet and Gelinas, 1999). Basal levels of NF-κB are detected in normal prostatic epithelial cells and the androgen-dependent CaP cell line LNCaP (Palayoor *et al*., 1999; Huang *et al*., 2001). It has been reported that crosstalk occurs between NF-κB signaling and steroid receptor signaling pathways (Palvimo *et al*., 1996; McKay and Cidlowski, 2000). We show that treatment of LNCaP

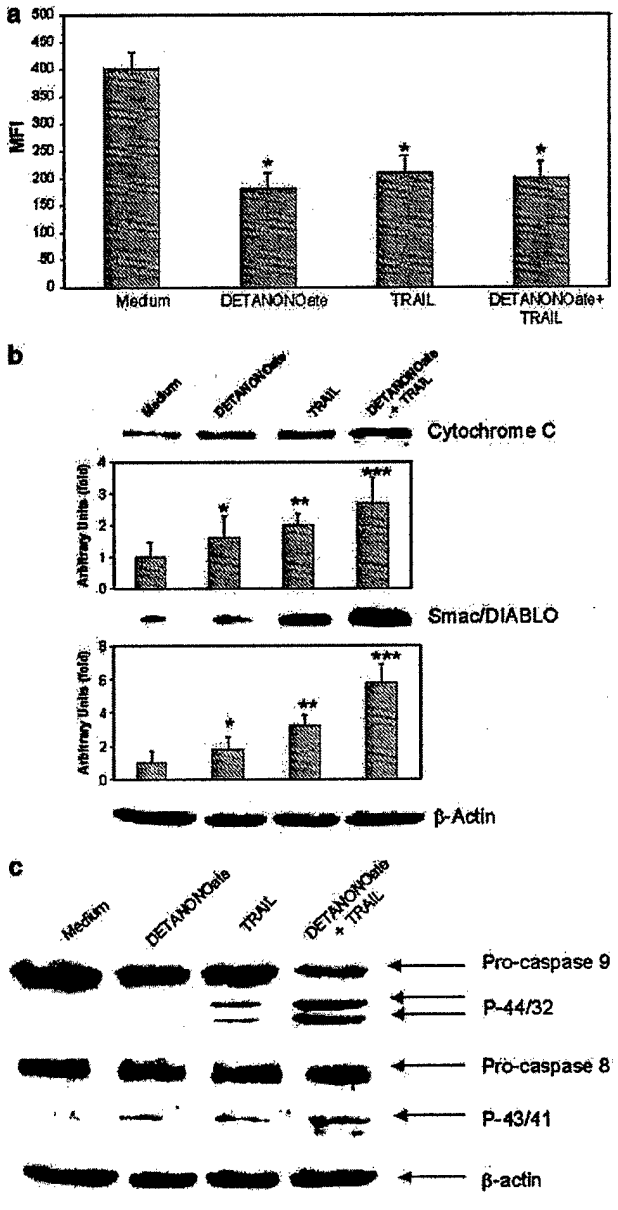

Figure 5 Mitochondrial membrane depolarization, release of cytochrome *c* and Smac/DIABLO into the cytosol, and activation of caspases 8 and 9. (**a**) Mitochondrial membrane activation. PC-3 cells were grown in FBS-free medium and treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. The PC-3 cells were then stained with DiOC6 and then analysed by flow cytometry. The findings demonstrate that DETANONOate, TRAIL, and the combination induce significant mitochondrial depolarization. The data represent the mean fluorescence intensity (MFI), and are the mean of three independent experiments. *$P<0.05$, medium *vs* cells treated. (**b**) Release of cytochrome *c* and Smac/DIABLO. PC-3 cells were grown in FBS-free medium and were treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted from the culture. The purified fraction of cytosolic protein was separated by SDS–PAGE and transferred onto the nitrocellulose membrane as described in Materials and methods. The membrane was stained with polyclonal anti-human cytochrome *c* antibody (top panel) or anti-Smac/DIABLO antibody (bottom panel). The blots represent one of two separate experiments. The data show that DETANONOate and TRAIL induce some release of both cytochrome and Smac/DIABLO, and the combination releases higher levels. The relative cytochrome *c* and Smac/DIABLO expression was determined by densitometric analysis of the blot. *$P<0.05$, **$P<0.03$, ***$P<0.002$ medium *vs* cells treated. (**c**) Activation of caspases 8 and 9. PC-3 cells were treated as described above. The activation of caspases 8 and 9 was determined by Western blot. There was some activation of caspase 8 by DETANONOate and some activation of caspase 9 by TRAIL. However, the combination resulted in significant activation of both caspases

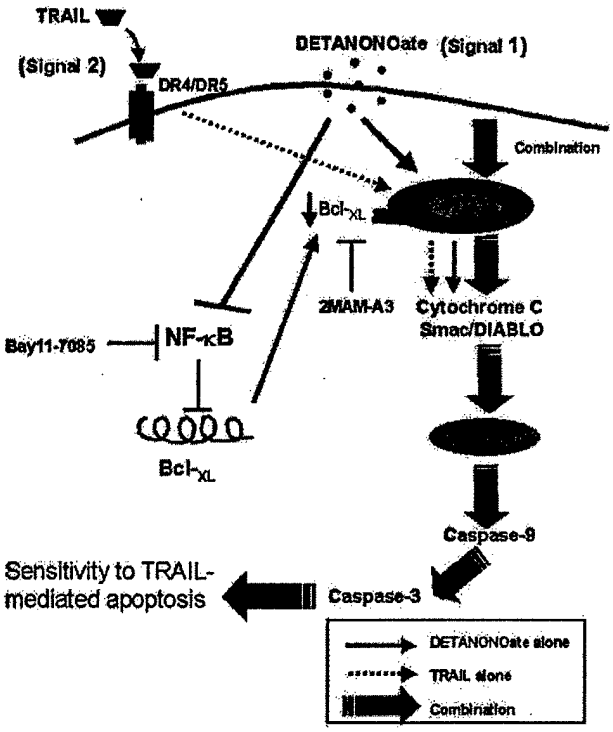

Figure 6 Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL. This figure schematically demonstrates that treatment of PC-3 cells with DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by DETANONOate, which partially inhibits NF-κB activity, and this leads to downregulation of Bcl-$_{xL}$ transcription. In addition, DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome *c* and Smac/DIABLO into the cytosol in the absence of downstream activation of caspase 9. Signal 2 is provided by TRAIL, which also partially activates the mitochondria with some release of cytochrome *c* and Smac/DIABLO in the absence of caspase 9 activation. However, the combination treatment results in significant activation of the mitochondria and release of high levels of cytochrome *c* and Smac/DIABLO, activation of caspases 9 and 3, resulting in apoptosis. The two-signal model is corroborated by the use of specific inhibitors in which inhibition of NF-κB by Bay11-7085 was sufficient to sensitize the CaP cells to TRAIL-induced apoptosis concomitant with downregulation of Bcl-$_{xL}$ expression. The role of Bcl-$_{xL}$ in the regulation of TRAIL apoptosis was corroborated by the use of the chemical inhibitor 2MAM-A3, which also sensitized the cells to apoptosis

with DHT sensitized the cells to TRAIL via inhibition of NF-κB. In contrast, androgen-independent CaP cells PC-3 and DU-145 have elevated NF-κB activity and this was confirmed here (data not shown). In addition, PC-3 and DU-145 cells have constitutively active IκB kinase complex (IKK), which activates NF-κB (Gasparian *et al.*, 2002). Thus, constitutive activation of NF-κB plays a central role in the resistance to CaP cell line to therapeutic agents.

The present findings demonstrate that DETANONOate inhibits NF-κB activity. It has been shown that high levels of NO inhibit NF-κB activity by several mechanisms. For instance, DETANONOate inhibits the phosphorylation and subsequent degradation of IκB-α, which prevents nuclear localization of NF-κB (Katsuyama *et al.*, 1998). Also, NO may quench reactive oxygen species that are responsible for the activation of NF-κB (Garban and Bonavida, 2001b). In addition, recent studies demonstrate that NO induces S-nitrosylation of NF-κB p50 and reduces its DNA-binding activity (Connely *et al.*, 2001; Marshall and Stamler, 2001). NF-κB displays redox-sensitive DNA-binding activity (Chinenov *et al.*, 1998; Tell *et al.*, 1998). This redox sensitivity is conferred by a single cysteine residue within the DNA-binding site (Matthews *et al.*, 1993; Marshall and Stamler, 2001). In this study, we demonstrate that NF-κB binding activity was significantly decreased after treatment with DETANONOate (Figure 2a). We also demonstrate that DETANONOate induced strongly S-nitrosylation of NF-κB p50 (Figure 2b) in agreement with the findings of Marshall and Stamler (2001) and Connely *et al.* (2001).

Recent studies demonstrated that Bcl-2 and Bcl-$_{xL}$ block apoptosis induced by physiological agents such as TRAIL in PC-3, DU-145, and LNCaP CaP cells (Rokhlin *et al.*, 2001). In addition, overexpression of Bcl-$_{xL}$ in LNCaP and PC-3 cells desensitized the cells to the effects of cytotoxic chemotherapeutic agents (Li *et al.*, 2001). However, downregulated endogenous levels of Bcl-$_{xL}$, but not Bcl-2, induced a marked increase in chemosensitivity (Lebedeva and Stain, 2000). These results suggest the important role of Bcl-$_{xL}$ in the resistance to apoptosis induced by cytotoxic agents like TRAIL in CaP. It is noteworthy that our results demonstrate that DETANONOate treatment induces selective downregulation of Bcl-$_{xL}$ expression and sensitizes the CaP cells to TRAIL-induced apoptosis. Further, inhibition of Bcl-$_{xL}$ function by 2MAM-A3 sensitizes the cells to TRAIL apoptosis. These findings corroborate the role of Bcl-$_{xL}$ in the regulation of resistance of CaP to chemotherapy and TRAIL.

The mechanism by which NO induces inhibition of Bcl-$_{xL}$ expression was examined. Previous findings demonstrated that the Bcl-$_{xL}$ promoter contains an element that binds NF-κB transcription factors and supports transcriptional activation by members of this family (Lee *et al.*, 1999). It was possible that DETANONOate inhibits NF-κB and this, in turn, inhibits Bcl-$_{xL}$ transcription. We demonstrate here that DETANONOate inhibits Bcl-$_{xL}$ expression via inactivation of NF-κB activity. This was shown by using a luciferase reporter construct driving the Bcl-$_{xL}$ promoter. Treatment with DETANONOate or Bay 11-7085 (which selectively and irreversibly inhibits the induced phosphorylation of IκB without affecting the constitutive IκB-α phosphorylation; Pierce *et al.*, 1997) significantly inhibited the high constitutive luciferase activity. However, there was little luciferase activity following the removal of the putative NF-κB-binding sequence from the promoter and neither DETANONOate nor Bay 11-7085 had any effect. These results directly demonstrate that Bcl-$_{xL}$ expression in PC-3 is primarily regulated by

NF-κB and inhibition of NF-κB, in turn, inhibits Bcl-$_{xL}$ transcription.

NO, synthesized from L-arginine by NO synthase, is a small, diffusible, highly reactive molecule with dual regulatory roles under physiological and pathological conditions (Schmidt and Walter, 1994). NO can promote apoptosis (proapoptosis) in some cells, whereas it inhibits apoptosis (antiapoptosis) in other cells. This dichotomy depends on the rate of NO production and the interaction with biological molecules such as iron, thiol, proteins, and reactive oxygen species (Schmidt, 1992; Stamler, 1994). High concentrations of NO and also long-lasting production of NO such as by DETANONOate used here act as proapoptotic modulators (Messmer and Brune, 1996; Poderoso *et al.*, 1996; Jun *et al.*, 1999; So *et al.*, 1998; Di Nardo *et al.*, 2000). The present findings are consistent with the proapoptotic effects of the high levels of NO used to sensitize CaP cells.

NO binds to cytochrome *c* oxidase (complex IV) in the mitochondrial electron transfer chain (Poderoso *et al.*, 1996). Under this condition, superoxide generated from mitochondria interacts with NO to form peroxynitrite, which induces mitochondrial dysfunction and cytochrome *c* release. NO also generates ceramide, which induces cytochrome *c* release from mitochondria (Ghafourifar *et al.*, 1999). Our results clearly show that DETANONOate induces activation of the mitochondria pathway, including mitochondrial membrane depolarization (Figure 3a) and some release of both cytochrome *c* and Smac/DIABLO (Figure 3b). The participation of the mitochondria is not complete because we demonstrate that downstream caspases are not activated. Caspase activation, however, resulted from the combination of DETNONOate and TRAIL. Recent studies have shown that caspase 8 activation is necessary but not sufficient for TRAIL-mediated apoptosis in prostate carcinoma cells (Rokhlin *et al.*, 2002), suggesting the important participation of the mitochondria-dependent pathway in TRAIL-mediated apoptosis. Further, our findings with DETANONOate are consistent with those of Lee *et al.* (2001), who reported that sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway.

This study demonstrates that the combination of NO donor and TRAIL can sensitize TRAIL-resistant CaP to TRAIL-induced apoptosis. This combination treatment is a result of two complementary signals induced by each agent alone (Ng and Bonavida, 2002a; schematically diagrammed in Figure 6). Signal 1 results from NO-induced perturbation of the mitochondria, inhibition of NF-κB activity, and downregulation of Bcl-$_{xL}$ expression. Signal 1 alone is not sufficient to promote the cells toward apoptosis. Signal 2 is induced by TRAIL, which activates the mitochondria slightly, but not sufficient to activate the apoptosome and induce apoptosis. However, combination of the two signals results in complementation and activation of the mitochondrial pathway and activation downstream of caspases 9 and 3 resulting in apoptosis. Thus, the findings of this report reveal that NO can selectively inhibit the expression of the antiapoptotic resistant factor Bcl-$_{xL}$ via inhibition of NF-κB activity. The findings also reveal new targets for intervention affecting NF-κB activity or Bcl-$_{xL}$ expression and whose modification may revert resistance of CaP to TRAIL apoptosis. Thus, NO donors or Bcl-$_{xL}$ inhibitors may be useful in the treatment of TRAIL-resistant tumors in combination with TRAIL or TRAIL agonists such as antibody against DR4/DR5 (DR: death receptor) (Ichikawa *et al.*, 2001).

Materials and methods

Reagents

The anti-Bcl-$_{xL}$ and anti-β-actin monoclonal antibodies were purchased from Santa Cruz (California, USA) and from Calbiochem (San Francisco, CA, USA), respectively. mAb anti-Bcl-2 was obtained from DAKO Corporation (Carpinteria, CA, USA). The polyclonal antibodies anti-XIAP, anti-IAP-1, anti-IAP-2, anticaspase 8, anticaspase 9, and survivin were obtained from Cell Signaling (San Diego, CA, USA), anti-cytochrome *c* from Pharmingen (San Diego, CA, USA), and anti-Smac/DIABLO from Alexis (San Diego, CA, USA). The human recombinant TRAIL and TNF-α were obtained from PeproTech Inc. (Rocky Hills, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-active caspase 3 and FITC-conjugated IgG were purchased from Pharmingen (San Diego, CA, USA). The NF-κB inhibitor Bay 11-7085 (specific inhibitor of IκBα phosphorylation; Pierce *et al.*, 1997) was obtained from Calbiochem (San Francisco, CA, USA), and the Bcl-$_{xL}$ inhibitor 2MAM-A3 (binds to the hydrophobic groove of Bcl-2 and Bcl-$_{xL}$) (Tzung *et al.*, 2001) was obtained from Biomol (Plymouth, PA, USA). The DETANONOate was obtained from Alexis (San Diego, CA, USA).

Cells and culture conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent LNCaP and the androgen-independent (Tso *et al.*, 2000) CL-1 (LNCaP-derived) cell lines were kindly provided by Dr Arie Belldegrun at UCLA. Cells were maintained as a monolayer in 80 mm^2 plates in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids. FBS (Life Technologies) was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/l R1881 methyltrienolone (New Life Science Products, Boston, MA, USA). The cell cultures were maintained as monolayers on plastic dishes and were incubated at 37°C and 5% carbon dioxide in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated FBS (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to treatments.

Cell treatments

Log-phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6×10^4 cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1, and PC-3 cells were synchronized by treatment with 1% FBS for 18 h prior to each experiment. The treatment of NCaP cells was in a medium with 1% of serum and the treatments of DU145, CL1, and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate, or the combination for 18 h. For the experiments of sensitization to TRAIL-mediated apoptosis by the NF-κB inhibitor Bay 11-7085, the cells were treated with different concentrations of Bay 11-7085 for 1 h and then treated with various concentrations of TRAIL for 18 h. For sensitization to TRAIL-mediated apoptosis by the Bcl-$_{xL}$ inhibitor 2MAM-A3, the cells were treated with different concentrations of 2MAM-A3 for 4 h, and then treated with TRAIL for 18 h.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice-cold 1 × phosphate-buffered saline (PBS) and were resuspended in 100 μl of the cytofix/cytoperm solution (Pharmingen, San Diego, CA, USA) for 20 min. Thereafter, the samples were washed twice with ice-cold 1 × perm/wash buffer solution (Pharmingen) and were stained with FITC-labeled anti-active caspase 3 mAb for 30 min (light protected). The samples were subsequently washed once with 1 × perm/wash buffer solution and 250 μl of 1 × PBS was added prior to flow cytometry analysis on a flow cytometer EPICS^R XL-MCL (Coulter, Co. Miami, FL, USA), with the System II™ Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50)

The S-nitrosylation of NF-κB p50 was analysed by immunoprecipitation assay. The cells were grown in the presence and absence of DETANONOate (0, 500, and 1000 μM) and then harvested and pelleted at 14 000 *g* for 2 min. The resulting cell pellets were resuspended and dissolved in 500 μl ice-cold components of radioimmunoprecipitation assay (RIPA) buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2 μg of rabbit anti-S-nitrosylated proteins polyclonal Ab (Calbiochem, San Diego, CA, USA) and were subsequently incubated with 30 μl Immuno-Pure Plus Immobilized protein A (Lindmark *et al.*, 1983) (Pierce, Rockford, IL, USA) for 4 h at 4°C on a shaking platform. The lysates were centrifuged for 1 min at 14 000 *g* and the supernatants were discarded. The immunoprecipitates were washed twice with 1.0 ml of ice-cold RIPA buffer prior to assay. The immunoprecipitates were resolved on a 12% SDS–PAGE gel and subsequently immunoblotted with anti-NF-κB p50 polyclonal Ab (1 : 2000 dilution) (Active Motif, Carlsbad, CA, USA). The immunostaining was visualized by autoradiography.

Luciferase Bcl-$_{xL}$ promoter reporter assay

The Bcl-$_{xL}$ WT promoter luciferase (Bcl-x WT promoter) reporter plasmid and the Bcl-$_{xL}$ promoter missing the NF-κB-binding sequence (Bcl-x κB promoter) have been previously characterized (Lee *et al.*, 1999). PC-3 cells were transfected by electroporation using pulses at 250 V/975 μF (Bio-Rad), with 20 μg of Bcl-x WT promoter or Bcl-x κB promoter. After transfection, the cells were allowed to recover overnight and were cultured in six-well plates. Cells were treated with the specific NF-κB inhibitor Bay 11-7085 (2 or 3 μM), NO donor DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml) for 18 h. Cells were then harvested in 1 × lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate.

Measurement of mitochondrial membrane depolarization

The mitochondria-specific dye 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (Molecular Probes Inc., Eugene, OR, USA) was used to measure the mitochondrial potential. PC-3 cells were grown in six-well plates and were treated with TRAIL (2.5 ng/ml) and/or DETANONOate (1000 μM) simultaneously. After treatments, the cells were collected at 18 h. A total of 50 μl of 40 μM ($DiOC_6$) was loaded to stain the cells for 30 min immediately after the cells were collected. The cells were detached by using PBS supplemented with 0.5 μM ethylenediaminetetraacetic acid (EDTA), washed twice in PBS, resuspended in 1 ml of PBS, and analysed by flow cytometry as reported (Ng *et al.*, 2002).

Western blot analysis

PC-3 cells were cultured at a low FBS concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with TRAIL (2.5 ng/ml), DETANONOate (1000 μM), or the combination. The cells were then lysed at 4°C in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN, USA). Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). An aliquot of total protein lysate was diluted in an equal volume of 2 × SDS sample buffer, 6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 10 min. The cell lysates (40 μg) were then electrophoresed on 12% SDS–PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi *et al.*, 2001). Levels of β-actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) using the public domain NIH Image J Program (also available via the internet).

Isolation of cytosolic fraction and determination of cytochrome c and Smac/DIABLO content

PC-3 cells were grown under the conditions explained for Western blot. At the end of the incubation period, the cells were recovered with 1 × PBS/EDTA, washed with 1.0 × PBS/0.1% BSA and resuspended in two volumes of homogenization buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM 1,4-dithiothreitol (DTT), one tablet of Complete Mini protease inhibitor cocktail in 250 mM sucrose medium). After 30 min on ice, the cells were disrupted by 40 strokes of a dounce glass homogenizer using a loose pestle (Bellco Glass Inc., Vineland, NJ, USA). The homogenate was centrifuged at 2500 *g* at 4°C

for 5 min to remove nuclei and unbroken cells. The mitochondria were pelleted by spinning the homogenate at 16 000 *g* at 4°C for 30 min. The supernatant was removed and filtered through 0.1 μm Ultrafree MC filters (Millipore) to obtain the cytosolic fraction and was spun down at 16 000 *g* at 4°C for 15 min. The protein concentration of the supernatant was determined by the DC assay kit and was mixed with 2× Laemmli sample buffer and analysed by SDS–PAGE for determination of cytochrome *c* and Smac/DIABLO contents in the cytosolic fraction as previously reported (Jazirehi *et al.*, 2003).

Nuclear extracts preparation

Nuclear extract preparations were carried out as previously described by our laboratory (Garban and Bonavida, 2001b). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA, USA). After washing, cells were lysed in 1 ml of NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) on ice for 5 min. Samples were centrifuged at 300 *g* at 4°C for 5 min. The pellet was washed twice in NP-40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN, USA). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at −80°C.

EMSA

Nuclear proteins (5 μg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB using EMSA Kit Panomics™ (Panomics Inc., Redwood City, CA, USA) following the manufacturer's instructions (Vega *et al.*, 2004). A measure of 10 μl was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide sequences for NF-κB are as follows: 5′-AGTTGAGGGGACTT TCCCAGGC-3′ (Harada *et al.*, 1994). Relative concentrations were assessed by densitometric analysis as mentioned above.

Isobologram analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/DETONONOate combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5, and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 μM) as described (Berenbaum, 1978). Combinations yielding a cytotoxicity of 30±5% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose–response curves using active caspase 3 analysis for LNCaP, DU145, CL-1, and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18 h.

Statistical analysis

The experimental values were expressed as the mean±s.d. for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's *t*-test was used for comparison between two groups. Significant differences were considered for probabilities <5% ($P<0.05$).

Abbreviations

Bcl-$_{xL}$, Bcl-2 related gene; CaP, prostate cancer; DETANONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate; DHT, 5-α dihydrotestosterone; DR, death receptor; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IAP, inhibitor of apoptosis protein; IKK, IκB kinase complex; JNK, c-Jun N-terminal kinase; 2MAM-A3, 2-methoxyantimycin A_3; NF-κB, nuclear factor κB; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; RIPA, radioimmunoprecipitation assay (buffer); SDS, sodium dodecyl sulfate; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbolacetate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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Regulation of prostate cancer sensitivity to apoptosis by CDDP by downregulation of XIAP and Bcl-$_{xL}$ expression via inhibition of constitutive NF-κB activity

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Prostate cancer patients develop chemoresistance following initial treatment. Several studies have been addressed to determine the underlying mechanisms of resistance and included the development of the MDR phenotype, dysregulation of the apoptotic signaling pathways, etc. We and others have demonstrated in CaP cell lines that NF-κB is constitutively activated and regulates survival and growth of the tumor cells. We have demonstrated that PC-3 and DU-145 synthesize and secrete cytokines such as TNF-α and IL-6 that regulate NF-κB activity via an autocrine paracrine pathway. Further, these cytokines were found to regulate drug resistance. Therefore, we hypothesized that NF-κB may regulate drugs-induced apoptosis via dysregulation of the apoptotic signaling pathways and hence inhibition of NF-κB may chemosensitize the cells. The objective of this study was to test this hypothesis and delineate the gene products that are transcriptionally regulated by NF-κB and contribute to chemoresistance. We demonstrate that treatment of PC-3 cells with NF-κB inhibitors sensitized the cells to CDDP-induced apoptosis and synergy was achieved. Further, inhibition of NF-κB by a nitric oxide donor (DETANONOate), via the S-nitrosylation of p50, also resulted in chemosenstization. The effect of NF-κB on the apoptosis signaling pathways, as determined by western, revealed that there was selective downregulation of the anti-apoptotic XIAP and Bcl-$_{xL}$ gene products following inhibition of NF-κB. The role of these proteins in chemosensitization was corroborated by the use of specific inhibitors. These findings suggest that tumor cells develop a self-contained mechanism to resist drug-induced apoptosis by tumor-derived autocrine/paracrine factors which activate NF-κB and secure cell growth and survival. Further, the present findings suggest that NF-κB, XIAP and Bcl-$_{xL}$ are targets for intervention in the reversal of drug resistance. Supported by a grant from the Department of Defense (US Army DAMD 17-02-1-0023), Jonsson Comprehensive Cancer Center (MV, AJ), UCLA SPORE in Prostate Cancer (P50 CA92131-01A1), Fogarty (SH-Y, MV, HG), and UC MEXUS (SH-Y).

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